The effect of endogenous and exogenous chemicals on drug metabolizing enzymes and drug transporters in human hepatocytes

by

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Preface

Significant variability in the pharmacokinetics of drugs such as cyclosporine, tacrolimus, sirolimus and mycophenolic acid, is seen in liver transplant patients. These agents are primarily metabolized by CYP3A4 or UGT1A1, and are also substrates for drug transporters such as P-glycoprotein, multidrug resistance protein 2 (MRP2) and bile salt export pump (BSEP). Factors modulating the expression and activity of these enzymes and transporters will lead to changes in the clearance of immunosuppressive agents. Inflammation associated with infection or organ rejection after transplantation can modulate the expression and activity of CYP3A4, UGT1A1 and various drug transporters. HIV-protease inhibitors (HIV-PIs) used for the treatment of HIV infection are shown to modulate the blood levels of immunosuppressive agents.

The primary goal of this dissertation research was to evaluate the effect of endogenous chemicals such as cytokines and exogenous compounds such as HIV-protease inhibitors on human hepatic drug metabolizing enzymes and transporters using primary cultures of human hepatocytes.

The results from this research indicate that HIV-protease inhibitors generally decrease CYP3A4 activity inspite of increasing CYP3A4 mRNA and protein. The effect of PIs on CYP3A4 recovers over time. PIs also increased UGT1A1 mediated metabolism as well as mRNA expression of transporters. These effects are dependent on the HIV-protease inhibitor

used and its concentration. Additionally, HIV-PIs increased the expression and activity of hepatic efflux transporters. These effects of HIV-PIs are found to be potentially mediated through alteration in PXR and CAR expression. Cytokines, released during infection or inflammation process, were observed to downregulate the expression and activity of UGTs and transporters through modulation of PXR and CAR.

As compared to cytokines, the effect of HIV-protease inhibitors on drug metabolizing enzymes and transporters contributes significantly towards the variability in pharmacokinetics of immunosuppressive agents.

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1.1. Hepatic drug metabolism

Liver is the major organ involved in the process of drug metabolism, although some metabolism also occurs in the gut wall, lungs and kidneys. Hepatocytes contain the enzymes necessary for the metabolism of drugs and endogenous chemicals. Overall, the metabolic processes convert drugs into more water-soluble compounds by increasing their polarity. This is an essential step before the drug can be excreted in bile or urine (Woolf, 1999). Conventionally, drug metabolism is broadly divided into phase I and phase II metabolism processes. Some drugs may undergo just phase I or phase II metabolism, but more often drugs undergo phase I and then phase II metabolism sequentially.

Phase I metabolism includes processes such as oxidation, reduction, hydrolysis and hydration which result in the formation of functional groups (-OH, -SH, $-NH_2$ or –COOH) making the metabolite more polar than the parent compound (Gibson and P, 2001). Cytochrome P450 (CYP) is the most important phase I enzyme and refers to a superfamily of heme-containing enzymes, located in the membrane of endoplasmic reticulum of the cell and is responsible for the metabolism of a variety of xenobiotics and endobiotics (Woolf, 1999). Human CYP isoforms involved in the metabolism of xenobiotics include CYP1A1/2, CYP2B6, CYP2C8/9/19, CYP2D6, CYP2E1 and CYP3A4/5, of which CYP3A is the most abundant isoform (30 % of the hepatic CYPs) in human livers (Shimada et al., 1994; Eagling et al., 1998; Woolf, 1999). Functionally, CYP3A is responsible for the metabolism of about 53% of commonly prescribed drugs, followed by CYP2D6, CYP2C, CYP1A2 and CYP2E1 accounting for 25%, 18%, 3% and 1% of drug metabolism, respectively (Shimada et al., 1994; Woolf, 1999). The subfamily of CYP3A includes CYP3A4, CYP3A5 and CYP3A7. CYP3A4 and

CYP3A5 are expressed in adult livers, while CYP3A7 is expressed mainly in fetal liver (Venkatakrishnan et al., 2001).

Phase II metabolism involves processes such as methylation, acetylation, sulfation, glutathione conjugation and glucuronidation (Woolf, 1999). Uridine diphosphate glucuronosyltransferases (UGTs) are the important phase II enzymes located in the membrane of endoplasmic reticulum. They are responsible for glucuronidation of endogenous and exogenous compounds, which involves the transfer of the glucuronic acid residue from uridine diphosphoglucuronic acid to hydroxy or carboxylic acid group of the compound (Mackenzie et al., 1997). To date, in humans, 16 different UGT isoforms have been identified, classified into 1A or 2B subfamilies (Tukey and Strassburg, 2000). Among the UGT1A family, UGT1A1 is the most important isoform involved in the glucuronidation of bilirubin as well as estradiol, acetaminophen and irinotecan (Cheng et al., 1998; Court et al., 2001; Tukey et al., 2002). UGT1A6 and UGT1A9 are other isoforms involved in the metabolism of catechols, acetaminophen and 4-methylumbelliferone (Court et al., 2001). Table 1 lists selected drug metabolizing enzymes along with their respective substrates, inducers and inhibitors.

Table 1. Substrates, inducers and inhibitors for common drug metabolizing enzymes and hepatic canalicular transporters

CYP3A4	UGT1A1	Hepatic canalicular transporters		
		MDR1	MRP2	BSEP
Substrates				
testosterone	estradiol	HIV protease inhibitors; amprenavir, ritonavir, indinavir, saquinavir	estradiol-17β-glucuronide	taurocholate
midazolam	bilirubin	tacrolimus	glutathione bilirubin conjugates	conjugated and unconjugated bile acids
erythromycin	acetaminophen	cyclosporine	SN-38	
tacrolimus	SN-38	digoxin	glyburide	
cyclosporine		rhodamine 123	etoposide	
Inducers				
rifampicin	phenobarbital	rifampicin	rifampicin	CDCA
phenobarbital	rifampicin		phenobarbital	
hyperforin	chrysin			
Inhibitors				
ketoconazole		cyclosporine	cyclosporine	cyclosporine
ritonavir			rifampicin	troglutazone
verapamil			glyburide	rifampicin

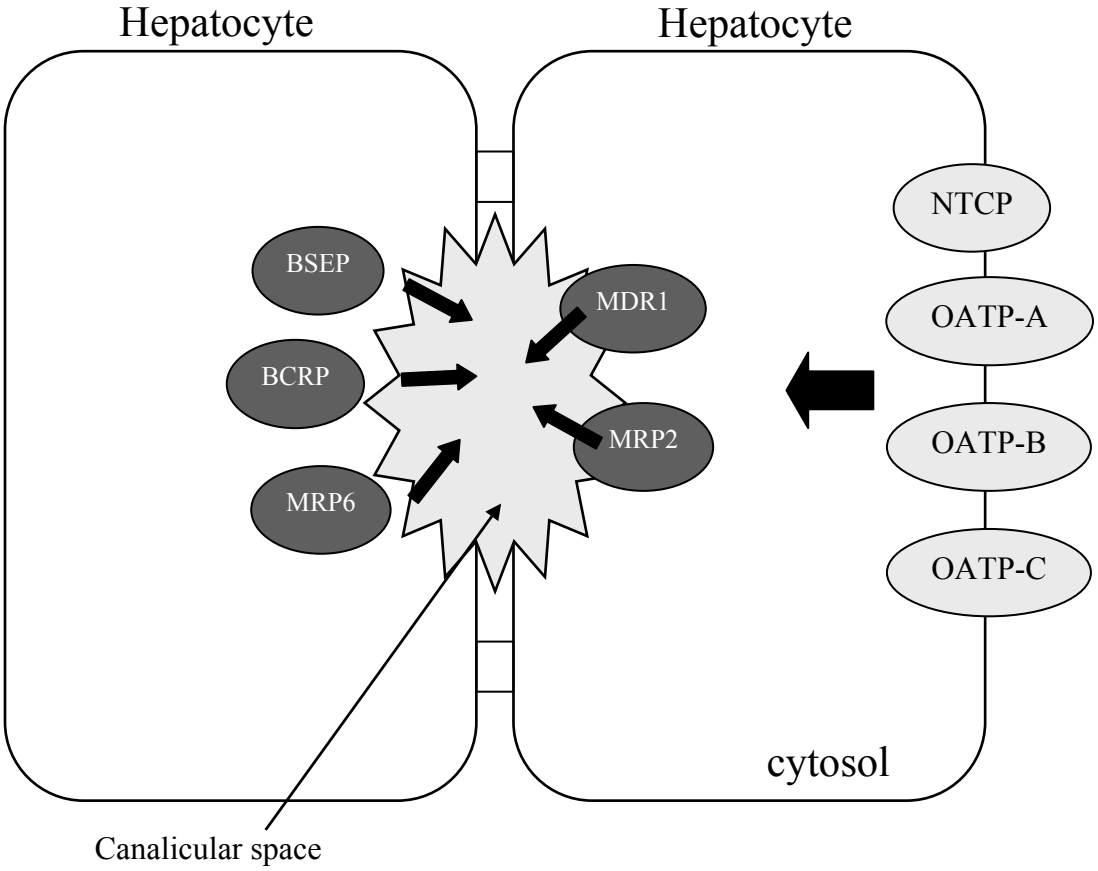
(Kostrubsky et al., 1999; Bowen et al., 2000; Sueyoshi and Negishi, 2001; Asghar et al., 2002; Chandra and Brouwer, 2004)

1.2. Hepatic drug transporters

Hepatobiliary transport and bile flow play important roles in the removal of endogenous and exogenous substances from the body, and adequate bile salt flow and recycling (Faber et al., 2003). Lipophilic molecules may move from plasma to hepatic cytosol by simple or facilitated diffusion. However, numerous transport proteins located in the sinusoidal (basolateral) membrane of the hepatocyte mediate uptake of amphipathic and polar organic compounds, as well as some lipophilic molecules, from sinusoidal plasma to hepatic cytosol, where these compounds are metabolized by the hepatic drug metabolizing enzymes. Hepatic transport proteins also play an important role in the excretion of drugs and metabolites from the hepatocyte into the canalicular space that exists between adjoining hepatocytes. Hepatic elimination is thus a sequence of events involving uptake of xenobiotice from the sinusoidal blood followed by intracellular metabolism and ultimately excretion into bile. Figure 1 shows a schematic of the uptake and efflux transporters in human liver and their localization in human hepatocytes. Table 1 lists selected drug transporters, their substrates, inducers and inhibitors.

Two classes of hepatic transporters are involved in the uptake and efflux of drugs, drug conjugates and endogenous substrates. They are classified as the solute carrier family (SLC) and the ATP binding cassette family (ABC). Na^+-taurocholate cotransporting polypeptide (NTCP, *SLC10A1*) is the basolateral transporter preferentially involved sodium dependent transport of conjugated bile acids (Karpen et al., 1996; Kouzuki et al., 2000; Chandra and Brouwer, 2004). Bromosulphthalein, estrone-3-sulphate are non bile salt substrates for this protein (Meier et al., 1997). The organic anion transporters (OATP, *SLC21* subfamily) are uptake carrier proteins that are involved in the Na-independent transport of a variety of structurally diverse compounds such

as bromosulphophthalein, glycocholate, prostaglandin E2 and estradiol-17β-glucuronide (Kullak-Ublick et al., 2001).



BSEP, bile salt export pump; BCRP, breast cancer resistance protein; NTCP, sodium taurocholate cotransporting polypeptide; OATP, organic anion transporting polypeptides; MDR1, multidrug resistance protein 1; MRP, multidrug resistance associated protein

Figure 1. Hepatic uptake and efflux transporters in human liver

Modified from: (Trauner et al., 1998; Faber et al., 2003; Chandra and Brouwer, 2004)

The biliary excretion of drugs and metabolites occurs predominantly by uni-directional ATP-dependent export pumps that transport substrates across canalicular membrane into bile. The canalicular membrane transporters that have been described to date are multidrug resistance

associated protein 2 (MRP2, *ABCC2*), bile salt export pump (BSEP, *ABCB11*), multidrug resistant protein 3 (MDR3, *ABCB4*), multidrug resistance protein 1 (MDR1, *ABCB1*) and breast cancer resistance protein (BCRP, *ABCG2*) (reviewed in (Faber et al., 2003)).

The most widely recognized canalicular transporter is MDR1, the multidrug resistance protein, P-glycoprotein. It primarily mediates the transport of large variety of substrates usually containing planar aromatic motifs and having molecular weight greater than 400 (Oude Elferink RP, 1995). In contrast to rodents, human hepatic expression of MDR1 is low compared to the intestine (Schuetz et al., 1995). MRP2 is responsible for the biliary secretion of organic anions such as acetaminophen glucuronide, camptothecin, SN-38, bile salts, glutathione, glucuronide and sulfate conjugates (Koike et al., 1997). MDR3 (*ABCB4*) plays an important role in phospholipids secretion. Patients classified with PFIC type 3 cholestasis exhibit mutations in *ABCB4* genes but the physiological role of MDR3 as drug transporter remains to be identified (Smith et al., 2000). BCRP is a 72-kDa ABC half- transporter and is thought to play an important role in the biliary excretion of sulfated conjugates of steroids and xenobiotics. BSEP is responsible for the excretion of conjugated and unconjugated bile salts, such as taurocholate, into the canalicular spaces (Gerloff et al., 1998). Though its role in cellular handling of xenobiotics is not yet clear, BSEP may be an important site of drug interactions resulting in hepatotoxicity. MRP6 (ABCC6) is another transporter with high expression levels in liver and kidneys but fail to show expression in other tissues (Kool et al., 1999). Alterations in these transporters, through inhibition or induction, can affect the intracellular concentration of drugs, resulting in altered pharmacokinetic and pharmacodynamic profiles, or of endogenous substances, altering normal physiological processes in the liver.

1.3. Regulation of hepatic drug metabolizing enzymes and drug transporters

Understanding of the regulation of hepatic drug metabolism and transporters is important to gain better knowledge of hepatic transport biology and facilitate predictions of how drugs and/or disease state that affect the intracellular regulatory mechanisms can alter hepatic metabolism and transport of exogenous and endogenous compounds. Mechanisms for regulation generally involve alteration in the function, expression or changes in the protein structure. Long-term modulation of expression can occur at several different levels such as transcription, translation and post translation. Transcription factors play an important role in the regulation of drug metabolizing enzyme and transporter gene expression in hepatocytes (Muller, 1998).

The regulation of drug metabolizing enzymes and transporters within the liver is complex and may involve multiple nuclear receptors that are able to converge on the same response element governing the expression of a single gene. Nuclear hormone receptors comprise a large superfamily of ligand activated transcription factors containing a conserved DNA-binding domain, a hinge region and a carboxy-terminal domain responsible for ligand binding and dimerization (Kumar and Thompson, 1999). Xenobiotics interact with these receptors, and the resulting complexes bind to the regulatory region of the gene to modulate its expression. Once a ligand binds to the receptor, the complex proceeds to bind to heterodimeric partner, retinoic X receptor (RAR), and this dimmer is then able to initiates the transcription. These transcription regulators serve to protect the liver against exogenous and endogenous toxic compounds. To date, several nuclear hormone receptor types have been reported to be involved in the transcriptional regulation of drug metabolizing enzymes and transport proteins and can roughly be divided into four classes: aryl hydrocarbon receptor (AhR), constitutive androgen receptor (CAR), pregnane X receptor (PXR) and the farnesoid X receptor (FXR). Figure 2 shows the

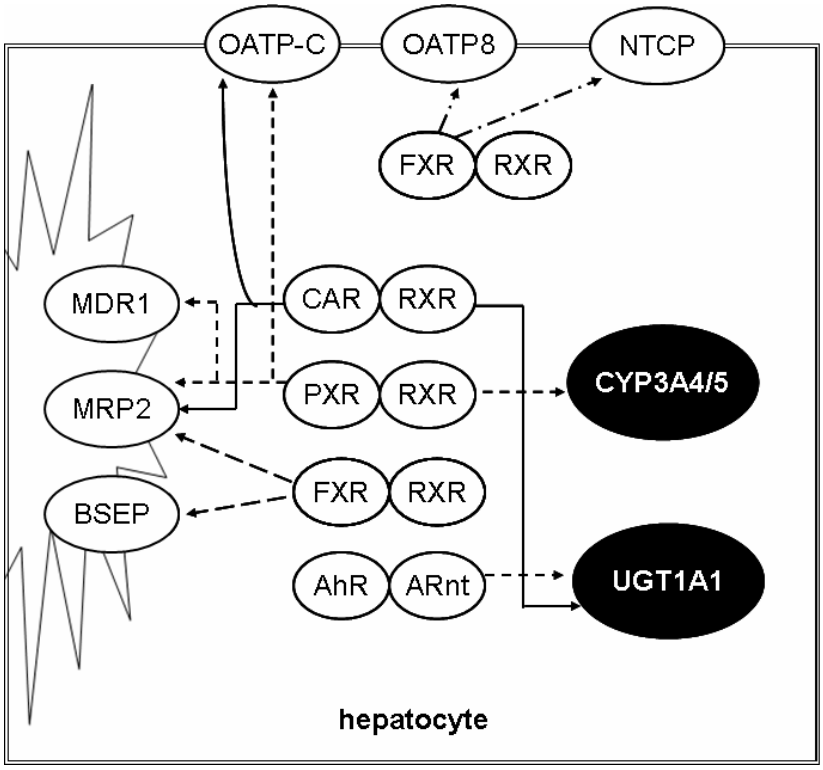
important nuclear receptors involved in the regulation of drug metabolizing enzymes and drug transporters in human liver.

The aryl hydrocarbon receptor (AhR) is a member of the PAS superfamily of transcription factors. It can be activated by a variety of chemicals including the aryl hydrocarbons, benzo(a)pyrene and 3-methylcholanthrene and the dietary plant constituents β-naphthoflavone and chrysin (Nebert and Gonzalez, 1987; Sogawa and Fujii-Kuriyama, 1997; Galijatovic et al., 2000). Following heterodimerization with the AhR nuclear translocator (ARNT), the AhR-ARNT complex translocates to the nucleus where it binds to specific dioxin response elements (DREs) with a defined core nucleotide sequence TNGCGTG (Dolwick et al., 1993; Lusska and Whitlock JP, 1993). While the mechanism for enzyme induction was first characterized for CYP1A, drug response elements (DREs) for AhR have also been identified in the promoter regions for human *UGT1A1* and *UGT1A6* (Munzel et al., 1998; Yueh et al., 2001).

PXR is the most extensively studied of the nuclear receptors and has been cloned in a number of species including rabbit, pig, monkey and human (Zhang et al., 1999; Kliewer and Willson, 2002). Human PXR has been shown to play an important role in the induction of CYP3A4, UGT1A1, UGT1A6, MDR1, MRP2, and OATP-C (Kliewer, 1998; Schuetz et al., 2001; Kast HR, 2002; Tirona et al., 2003).

The constitutive androgen receptor (CAR), as with other orphan nuclear receptors, also partners as a heterodimer with RXR to AGGTCA-based DNA response element (Sueyoshi and Negishi, 2001). It is predominantly expressed in the intestine and liver (Baes M, 1994). Human CAR modulates phenobarbital mediated induction of CYP3A4, UGT1A1, MRP2 and OATP-C (Sueyoshi et al., 1999; Sugatani et al., 2001; Goodwin B, 2002; Kast HR, 2002). Profound species differences in ligand specificity exist between human and rodent PXR as well as CAR

(Poland A, 1980; Maglich JM, 2003). Both PXR and CAR bind similar ligands, such as phenobarbital, rifampicin and dexamethasone and are thought to modulate gene induction through similar response elements (Sueyoshi et al., 1999; Moore LB, 2000; Xie W, 2000).



AhR, aryl hydrocarbon receptor; Arnt, AhR nuclear translocator, CAR, constitutive androgen receptor; FXR, farnesoid X receptor; PXR, pregnane X receptor; RXR, retinoid X receptor

Figure 2. Nuclear receptors involved in the regulation of hepatic drug metabolizing enzymes and transporters

Modified from: (Trauner et al., 1998; Faber et al., 2003; Chandra and Brouwer, 2004)

Farnesoid X receptor (FXR), after heterodimerizing with retinoid X receptor (RXR), is largely involved in the induction of transporters responsible for the efflux of bile salts in conditions of accumulation of potentially hepatotoxic bile acids and bile acid conjugates

(Ananthanarayanan M, 2001). The FXR-RXR heterodimer preferentially binds to the IR-1 element, consisting of an inverted repeat of AGGTCA hexamers, separated by one base pair, but has been shown to also bind to DR-3 and DR-4 motifs (Laffitte BA, 2000). FXR has been shown to induce BSEP, MRP2 along with OATP8 (Schuetz et al., 2001; Kast HR, 2002; Jung D, 2004). A summary of the above information is provided in Table 2.

Table 2. Nuclear receptors involved in the regulation of common hepatic drug metabolizing enzymes drug transporters

Nuclear receptor	Ligands	Some major target genes
PXR	Xenobiotics, Rifampicin, Ursodeoxycholic acid	CYP3A4, UGTs, MDR1, MDR3, MRP2, MRP4, BSEP
CAR	Xenobiotics, phenobarbital	CYP3A, UGTs, OATP-C, MRP2, MRP4, MDR1
FXR	Bile acids	BSEP, UGTs, MRP2, MDR3
VDR	Vitamin D, lithocholic acid	CYP3A
RXRα	9-cis-retinoic acid	Heterodimeric partner of all nuclear receptors

PXR, pregnane X receptor; CAR, constitutive androgen receptor; FXR, farnesoid X receptor; AhR, aryl hydrocarbon receptor; RXR, retinoid X receptor; VDR, vitamin D receptor.

1.4. Drug metabolism and transport in liver transplant patients

Liver transplantation is considered as the treatment of choice for patients with end-stage liver disease and in some metabolic liver diseases where standard medical or surgical therapeutic alternatives are not readily available. More than 5000 liver transplants are performed on an

annual basis in the US. Common indications for liver transplantation are cholestatic diseases (primary biliary cirrhosis), chronic hepatitis (hepatitis B, hepatitis C infections, and chronic drug toxicity), alcoholic cirrhosis, metabolic disorders (Wilson's disease, tyrosinemia, hemochromatosis) and hepatocellular carcinoma. Human immunodeficiency virus (HIV) infection was previously considered as an absolute contraindication to liver transplantation because of the high mortality rate due to opportunistic infection associated with HIV infection. However, the improvement in immune status and increased survival among individuals with HIV infection, which was made possible by the introduction of highly active antiretroviral therapy (HAART) in 1996, has made liver transplantation a possible treatment option even in patients who are HIV positive (Ragni et al., 2003; Neff et al., 2004).

Management of patients post liver transplantation is critical as many acute complications are common. These include allograft dysfunction, vascular complication involving hepatic artery thrombosis, and allograft rejection along with occurrence of bacterial and fungal infections. Long-term management of hypertension, diabetes mellitus, and hyperlipidemia is also frequently needed after liver transplantation. Thus, liver transplant patients are generally treated with multiple drugs that include one or more immunosuppressive drugs, antibiotics, antiviral drugs and antifungal drugs. Table 3 lists the drugs commonly used as part of a therapeutic regimen in transplant patients.

Importantly, the success of solid organ transplantation requires the use of immunosuppressive drugs that suppress the host response to an allograft enabling the transplantation of foreign organ into the recipient. Immunosuppression following transplantation is crucial to the survival of both allograft and patient. Major advancements in the field of immunosuppression involve introduction of cyclosporine in the early 1980s, followed by

tacrolimus and mycophenolic acid in the 1990s. During the late 1990s, a combination based approached using these immunosuppresive agents was introduced which was particularly effective in reducing graft rejection. For example, the combination of mycophenolic acid with cyclosporine and prednisone led to the reduction of acute rejection to 30-40 % in liver transplant patients. The most recent combination of immunosuppressive agents includes the use of low-dose tacrolimus and sirolimus with or without prednisone and mycophenolic acid (Wiesner et al., 2003).

1.5. Variability in pharmacokinetics of immunosuppressive agents

All the immunosuppressive agents currently used for prevention of rejection have a narrow therapeutic index. Under-dosing increases the risk of immunological rejection and the prospect of organ loss or damage, with potentially fatal consequences. Overdosing increases the risks associated with immunosuppression such as infection and malignant diseases, as well as drug specific adverse effects. These adverse effects can be serious such as development of diabetes mellitus with corticosteroids and calcineurin inhibitors (cyclosporine and tacrolimus), nephrotoxicity and hypertension with calcineurin inhibitors, and hyperlipidemia with sirolimus. Thus, it is extremely important to achieve and maintain target blood concentrations of the immunosuppressive agents during the treatment period.

Some important factors responsible for the intra- and inter- patient variability in the pharmacokinetics of the immunosuppressive drugs, thus requiring close monitoring and adjustments in their dosing regimen, are discussed briefly in the following section.

Table 3. Drugs commonly used in patients undergoing liver transplantation

Drug class	Drugs
Immunosuppressive agents	Prednisone Cyclosporine A Tacrolimus Sirolimus Mycophenolic acid
Antifungal agents	Ketoconazole Itraconazole Fluconazole Voriconazole
Antibiotics	Sulfamethoxazole-trimethoprim Acyclovir Ganciclovir
Antiviral agents	Ritonavir Lopinavir Nelfinavir Indinavir
Antihypertensive agents	Nifedipine Diltiazem

1.5.1. Ischemia /reperfusion injury and release of cytokines can alter drug metabolism

A period of cold ischemia is inevitable during the procurement of an organ for transplantation. On restoring the blood supply, the liver is subjected to warm reperfusion injury in the donor. This ischemia-reperfusion injury (I-R) leads to a multitude of conditions with the common clinical outcome of a poorly functioning graft (Delva et al., 1989; Huguet et al., 1992; Henderson, 1999; Serracino-Inglott et al., 2001). Experimental animal models of hepatic

ischemia-reperfusion injury have shed considerable light on the cellular and molecular mechanisms by which this insult produces a local acute inflammatory response that can, in some cases, lead to a systemic inflammatory response.

The initial phase initiated upon reperfusion that lasts for 2-3 hours is mediated by Kupffer-cell mediated production of reactive oxygen species (ROS) including superoxide anion and hydrogen peroxide. The severe injury that develops during the later phase (> 6 hours) is mediated primarily by activated neutrophils that accumulate in postischemic liver.

The hepatic inflammatory response to ischemia appears to begin with the elaboration of the cytokine, interleukin –12 (IL-12), which further leads to increased expression of tumor necrosis factor-alpha (TNFα) and interleukin –1 (IL-1). The cytokines interleukin – 6 (IL-6), interleukin –10 (IL-10) and interleukin –13 (IL-13) have been shown to be expressed during hepatic ischemia-reperfusion. Nitric oxide (NO) has also been shown to be another important regulatory mediator.

Use of immunosuppressive agents in the therapy helps in reducing the damage caused by ischemia – reperfusion. Cyclosporine and tacrolimus, reduce both tissue free radical levels and neutrophil infiltration after ischemia- reperfusion (Garcia-Criado et al., 1997). A new immunosuppressive agent FTY720 has been shown to prolong the damage due to ischemia and reperfusion. Though the release of cytokines after ischemia-reperfusion occurs over a comparatively short period of time, it can lead to systemic inflammatory response.

1.5.1.1. Pro-inflammatory cytokines - endogenous chemicals affecting drug metabolism and transporters

The inflammatory response, or the acute phase response (APR), is a systemic process in which the liver plays an important regulatory role. During tissue injury or infection, the body

reacts immediately with a series of specific physiological processes that eventually lead to the restoration of homeostasis. This APR starts within the liver where the Kupffer cells produce different pro-inflammatory cytokines, including tumor necrosis factor alpha (TNFα), interleukin (IL)- 1β and IL-6. In addition, various inflammatory transcription factors such as NF-κB and AP-1 are also activated.

Various reports have shown that IL-1, IL-6 and TNFα have suppressive effects on the expression and activity of different CYPs in *in-vivo* and *in-vitro* systems such as primary rat and human hepatocytes and microsomes. In addition, nitric oxide generated from hepatocytes and Kupffer cells in response to cytokines is able to react with heme-containing proteins such as CYP enzymes and can decrease CYP mediated activities. Nitric oxide is shown to inhibit expression of some CYPs, thus contributing towards down-regulation of CYPs during inflammation.

Compared to the studies describing the effects of cytokines on CYP expression and activity, there are limited reports published documenting the effect of cytokines or inflammation on phase II metabolizing enzymes such as UGTs. (Levesque et al., 1998) have reported IL-1α mediated inhibition of formation of dihydrotestosterone glucuronide in LNCaP cells. In rats, after injection of turpentine, the rate of p-nitrophenol glucuronidation (UGT1A6 marker) by hepatic microsomes remained unchanged, while the formation of testosterone glucuronide (UGT2B1/3/6 marker) was reduced to 65% of control. The mRNA expression of UGT1A1 and UGT2B3 was decreased after turpentine injection. The same study reported IL-6 (50 units/ml) mediated suppression of UGT1A1 and UGT2B3 mRNA expression in primary rate hepatocytes, with no effect of IL-1 on UGT mRNA levels (Strasser et al., 1998).

Hepatic mRNA expression of uptake transporters such as Oatp1, Oatp2, Oatp4 and Ntcp and that of efflux transporters such as Mrp2, Mrp6, Mdr1a, Bsep was reported to be decreased drastically 6 hours after LPS administration in rats. In contrast, LPS increased mRNA levels of Mrp1, Mrp3 and Mdr1b concurrently with the down-regulated transporters. Pretreatment with dexamethasone, which decreases the release of cytokines, reversed the reduction of Mdr1a, Oatp1, Oatp2 and Ntcp mRNA following LPS administration and also prevented the LPS-mediated increase in Mrp1, Mrp3 and Mdr1b mRNA expression, indicating the involvement of cytokines in mediating these responses (Cherrington et al., 2004).

IL-6 is believed to be involved in the reduction of mdr1a and mdr1b expression during acute inflammatory response in rats. Results have shown that reduction in mRNA levels of mdr1a and mdr1b observed *in vivo* in turpentine-induced acute inflammation are mediated primarily through a decrease in their gene transcription. Results from the *in vitro* studies in cultured rat hepatocytes demonstrated both an IL-6 mediated reduction in mRNA levels of mdr1a and mdr1b as well as IL-6 mediated suppression of mdr1a and mdr1b gene transcription (Sukhai et al., 2000). Fang et. al. have reported a decreased expression of mRNA expression of nuclear receptors RXRα, PXR, CRA, FXR and LXR , 4 hours after treatment of rats with LPS (Fang et al., 2004).

Study in rat hepatocytes showed that IL-1β and IL-6 have suppressive effects on the expression and activity of P-glycoprotein in cultured hepatocytes. Maximal inhibitory effect of IL-6 was observed after 24 hours, while maximum inhibition with IL-1β was observed after 72 hours of treatment, suggesting that IL-6 possibly acted by altering the mRNA expression of P-glycoprotein and IL-1β altering the protein synthesis and stability (Sukhai et al., 2001).

In rat hepatocytes, treatment with interferon (IFN-β) did not significantly affect the P-glycoprotein activity while interferon (IFN-γ) treatment decreased P-glycoprotein activity. The expression of P-glycoprotein however increased after IFN-γ, suggesting impaired maturation or dysfunction of the efflux transporter after IFN treatment (Akazawa et al., 2002).

There are very few studies published documenting the effect of LPS or individual pro-inflammatory cytokines on human drug transporters. Table 4 summarizes the effect of various cytokines on the expression and activity of human drug transporters. Elferrink et. al. have studied the effect of LPS on expression of various drug transporters in human liver slices. NTCP mRNA was downregulated and showed an inverse correlation with the amounts of TNFα and IL-1β produced. In contrast, MRP2 and BSEP mRNA levels were not affected 24 hours after LPS challenge, but both proteins were virtually absent in human liver slices suggesting that posttranscriptional mechanisms play an important role in LPS- induced regulation of human MRP2 and BSEP in liver slices (Elferink et al., 2004).

Table 4. Effect of cytokines on the expression and activity of human drug transporters

	NTCP		MDR1			MRP2			BSEP		Reference
	mRNA	Prot.	mRNA	Prot.	Act.	mRNA	Prot.	Act.	mRNA	Prot.	
LPS	↓[a]	↓[a]				↔[a]	↓[a]		↔[a]	↓[a]	(Elferink et al., 2004)[a]
IL-1β			↔[b]	↓[b]	↓[b]	↔[b],		↑[b]			(Lee and Piquette-Miller, 2003)[b]
						↓[c]	↓[c]				(Hisaeda et al., 2004)[c]
IL-2			↓[d]	↓[d]	↓[d]						(Stein et al., 1996)[d]
IL-6			↓[b]	↓[b]	↓[b]	↔[b]		↑[b]			(Lee and Piquette-Miller, 2003)[b]
			↑[e]								(Bertilsson et al., 2001)[e]
TNFα			↓[b]	↓[b]	↓[b]	↔[b]	↓	↑[b]			(Lee and Piquette-Miller, 2003)[b]
			↔[c]			↓[c]					(Hisaeda et al., 2004)[c]
IFNγ			↓[d]	↓[d]	↓[d]						(Stein et al., 1996)[d]
			↑[e]								(Bertilsson et al., 2001)[e]

a, Human liver slices (LPS = 100 μg/ml); b, Human hepatoma cells (HuH) and HepG2, IL-1β (0.2ng/ml), IL-6 (10 ng/ml) and TNFα (0.2ng/ml); c, HepG2 cells, IL-1β (1-20ng/ml) and TNFα (1-20ng/ml); d, HCT15 and HCT116 cells; e, $CaCo_2$ cells.

1.5.2. Pharmacokinetic drug-drug interactions contribute significantly to the variability in the pharmacokinetics of drugs in transplant patients

Pharmacokinetic drug interactions involve the effect of one drug on the absorption, metabolism, excretion or protein binding of another drug. Important pharmacokinetic interactions occur when immunosuppressants are prescribed in combination and with other medications. The troughs levels of mycophenolic acid (MPA, the active agent) in immunosuppressive regimens combining tacrolimus and mycophenolic mofetil (MMF) are higher than in regimens combining cyclosporine and MMF. Drugs such as ketoconazole, fluconazole, erythromycin, verapamil, and atorvastatin are reported to increase, while carbamazepine, phenobarbital, phenytoin, rifampicin and St. John's wort decrease trough levels of cyclosporine and tacrolimus. Clinically, the most significant pharmacokinetic interactions have been observed in HIV positive patients undergoing liver transplantation, where immunosuppressive agents are combined with HIV-protease inhibitors such as ritonavir, lopinavir and nelfinavair.

In the presence of nelfinavir (1.5 mg daily dose), the dose of tacrolimus had to be reduced to 0.5 mg weekly, which was 1/70 of the normal tacrolimus dose, in order to maintain the tacrolimus blood concentration in the desired range of 5-15 ng/ml (Schvarcz et al., 2000).

A profound interaction between nelfinavir and tacrolimus has been shown in HIV-positive liver transplant patients. A 10- to 50-fold decrease in tacrolimus dosage was required to maintain therapeutic concentrations of tacrolimus, in patients receiving nelfinavir (Jain et al., 2002c). The interaction between tacrolimus and Kaletra, a combination of ritonavir and lopinavir, was found to be more significant compared to that observed with the single protease inhibitor nelfinavir. After initiation of Kaletra for the HIV-positive patient after transplantation,

tacrolimus half-life was significantly increased (20 days) thus requiring discontinuation of tacrolimus for 3-5 weeks, in order to maintain the required tacrolimus blood concentrations (Jain et al., 2003). A similar interaction between sirolimus and nelfinavir was observed in a 40-year old HIV positive female after liver transplantation. Even with one fifth the recommended dose of nelfinavir (250 mg twice a day), a nine-fold increase in the sirolimus trough concentration, three-fold increase in peak concentration, and 60 % increase in the area under the concentration curve (0-24 hours) of sirolimus was observed in this patient, compared to patients not receiving nelfinavir (Jain et al., 2002a). To date, no other drug interaction between an immunosuppressant drugs and any other drug has been reported that results in such a drastic change in the pharmacokinetics of the immunosuppressant drug. A complete evaluation of the mechanism of such an interaction has not been carried out.

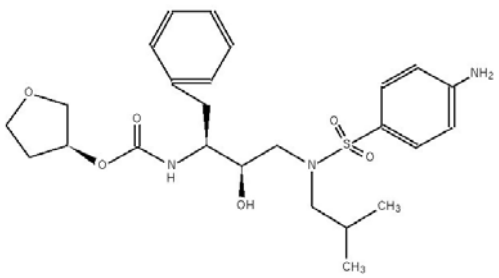
1.5.2.1. HIV protease inhibitors - exogenous compounds affecting drug metabolism and transporters

Inhibitors of human immunodeficiency virus (HIV)-encoded protease, combined with nucleoside analogues with antiretroviral activity cause profound and sustained suppression of viral replication, reduce morbidity and prolong life in patients with HIV infection.

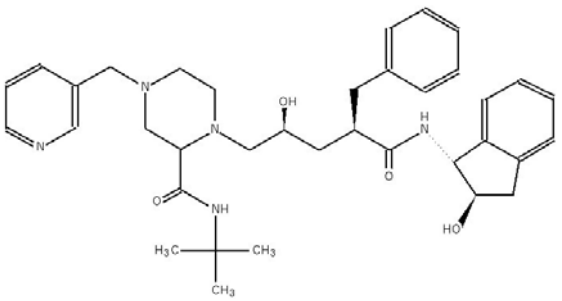
HIV protease inhibitors were first developed between 1989 and 1994 and were first introduced as treatment of HIV-1 infection in 1996, and are today considered part of standard therapy. Currently, there are seven HIV protease inhibitors approved by the FDA for the treatment of HIV infection. Amprenavir, atazanavir, indinavir, lopinavir, nelfinavir, ritonavir, saquinavir and the recently introduced tipranavir are the different HIV-protease inhibitors currently on the market. Chemical structures of the commonly used HIV-protease inhibitors are shown in Figure 3.

In vitro metabolism studies have shown that all the HIV protease inhibitors are predominantly metabolized by CYP3A enzymes with a minor contribution from CYP2D6 and CYP2C9 (Kumar et al., 1996; Chiba et al., 1997; Fitzsimmons and Collins, 1997).

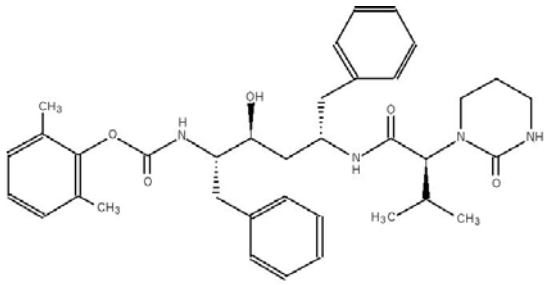
HIV protease inhibitors have complex effects on CYP3A family of enzymes. Studies done using rat or human microsomes and expressed enzyme systems have shown that HIV-protease inhibitors inhibit CYP3A4 enzyme to a varying degree. Ritonavir is by far the most potent inhibitor, followed by indinavir, nelfinavir, amprenavir and saquinavir in decreasing order of potency (Eagling et al., 1997; Decker et al., 1998; Iribarne et al., 1998; Koudriakova et al., 1998). Conversely, ritonavir and nelfinavir have also shown to induce CYP3A4 in studies carried out in rats and in human colon carcinoma cell lines (Greenblatt et al., 2000; Perloff et al., 2000; Venkatakrishnan et al., 2000; Venkatakrishnan et al., 2001). This induction phenomenon is of particular interest since all these protease inhibitors are used over an extended period of time in clinical practice. The induction of CYP3A4 by protease inhibitors may lead to decreased plasma drug levels of co-administered drugs that are substrates of CYP3A4, thus leading to loss of their therapeutic efficacy. Characteristics of commonly used HIV-protease inhibitors are listed in Table 5.



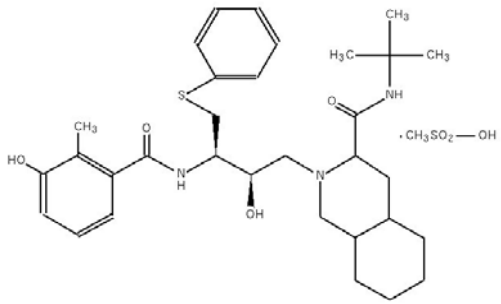
Amprenavir



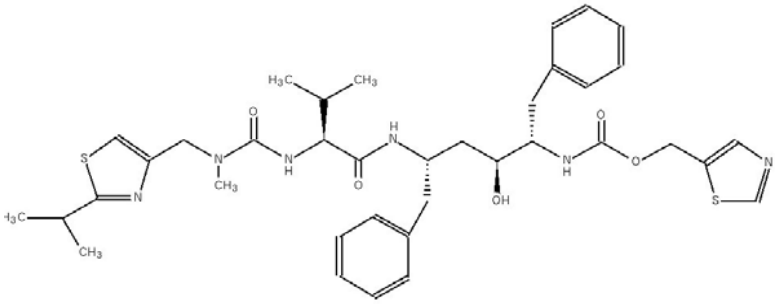
Indinavir



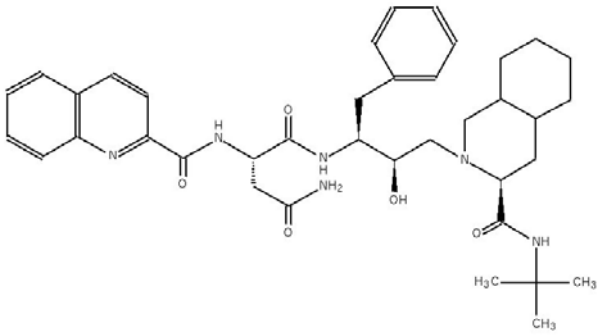
Lopinavir



Nelfinavir



Ritonavir



Saquinavir

Figure 3. Chemical structures of HIV-protease inhibitor

Table 5. Characteristics of commonly used HIV- protease inhibitors

Generic name	Form	Dosing	Oral bioavailability	Serum half-life (hours)	Route of metabolism	Adverse effects
Amprenavir (APV)	15 mg /ml oral solution	1400 mg two times/day	-	7.1- 10.6	CYP3A4	GI intolerance, nausea, vomiting Hyperlipidemia Hyperglycemia Transaminase elevation
Atazanavir (ATV)	100, 150 and 200 mg capsules	400 mg once daily	-	7	CYP3A4	Indirect hyperbilirubinemia Hyperglycemia Fat maldistribution
		RTV 100 mg + ATV 300 mg once daily				
Indinavir (IDV)	200, 333 and 400 mg capsules	800 mg every 8 hours	65%	1.5 - 2	CYP3A4	Nephrolithoasis GI intolerance, nausea, vomiting Hyperlipidemia Indirect hyperbilirubinemia
		RTV 100 or 200 mg + IDV 800 mg every 12 hours				

Generic name	Form	Dosing	Oral bioavailability	Serum half-life (hours)	Route of metabolism	Adverse effects
Lopinavir + Ritonavir (LPV + RTV)	Each capsule contains LPV 133.3 mg + RTV 33.3 mg Oral solution: Each 5 ml contains LPV 400 mg + RTV 100 mg	3 capsules or 5 ml two time daily	-	5 - 6	CYP3A4	GI intolerance, nausea, vomiting Fat maldistribution Hyperlipidemia (esp. hypertriglyceridemia) Hyperglycemia
Nelfinavir (NFV)	250 mg tablets or 625 mg tablets	1250 mg two times a day	20- 80 %	3.5 - 5	CYP3A4	GI intolerance, nausea, vomiting Fat maldistribution Hyperlipidemia Hyperglycemia
	50 mg/g oral powder	750 mg three times a day				
Ritonavir (RTV)	100 mg capsules or 600 mg /7.5 ml solution	600 mg every 12 hours (when used alone)		3 – 5	CYP3A4 CYP2D6	GI intolerance, nausea, vomiting Hyperlipidemia (esp. hypertriglyceridemia) Hepatitis Hyperglycemia

Generic name	Form	Dosing	Oral bioavailability	Serum half-life (hours)	Route of metabolism	Adverse effects
Saquinavir (SQV)	200 mg capsules or 500 mg tablets	RTV 100 mg + SQV 1000 mg two times/day	4% (when taken as sole PI- not generally recommended)	1 -2	CYP3A4 CYP2D6	GI intolerance, nausea, vomiting Fat maldistribution Hyperlipidemia (esp. hypertriglyceridemia) Hyperglycemia
Tipranavir (TPV)	250 mg capsules	RTV 200 mg + TPV 500 mg two times/day		6 hours after single dose of TPV/RTV	CYP3A4	Hepatotoxicity Fat maldistribution Hyperlipidemia (esp. hypertriglyceridemia) Hyperglycemia

1.6. Mechanism of variability in pharmacokinetics of immunosuppressive agents – role of drug metabolizing enzymes and transporters

It is now realized that the clinically significant variability in the pharmacokinetics of drugs can be explained by alterations in the metabolic enzymes and drug transporters that are present in the liver and extra-hepatic tissues. These alterations could be due to endogenous mediators such as cytokines or exogenous compounds such as co-administered drugs or due to genetic factors that result in changes in the enzyme itself, making it more or less functional.

Drug interactions involving metabolizing enzymes are generally of two types: enzyme induction, where metabolism of the substrate drug is increased and enzyme inhibition, where metabolism of the substrate drug is reduced, in the presence of co-administered drug. Pharmacokinetic drug interactions associated with immunosuppressive drugs can also be explained by changes in metabolizing enzymes and/or drug transporters, as most of the immunosuppressants used in therapy are substrates of cytochrome P450 (CYP) enzymes and various transporters. Table 6 lists the metabolizing enzymes and drug transporters involved in hepatic handling of immunosuppressive agents.

The major pathway of metabolism of cyclosporine, tacrolimus and sirolimus is via CYP3A4 and all these agents are also substrates for the efflux transporter, P-glycoprotein. Absorption of these drugs after oral administration is affected by both P-glycoprotein and CYP3A4 activity in the intestine, while the hepatic metabolism is dependent on hepatic CYP3A activity. Thus, drugs that are either inducers or inhibitors of CYP3A4 enzyme and P-glycoprotein can alter levels of these drugs by altering its metabolism or transport. Mycophenolate mofetil (MMF) is a pro-drug that is rapidly and almost completely absorbed from the gut where it is de-esterified to form mycophenolic acid (MPA), the active

immunosuppressant. MPA is converted by the uridine diphosphate glucuronyltransferase (UGT) family into 7-hydroxy-glucuronide mycophenolic acid (MPAG) which is excreted into bile and is not pharmacologically active. UGT1A9 is the main isoform involved, with at least 55 % contribution to the hepatic MPAG formation, with UGT1A1 and UGT1A6 accounting for a part of MPAG formation in liver. Studies carried out in Mrp2 deficient rats (Eisai hyperbilirubinemic rats) have shown that, multidrug resistant protein 2 (Mrp 2), an efflux transporter located on the apical side of hepatocytes, is involved in the biliary excretion of MPAG. Cyclosporine mediated inhibition of the biliary excretion of MPAG by the Mrp2 transporter is believed to be the mechanism responsible for the interaction between cyclosporine and MMF.

Table 6. Metabolizing enzymes and drug transporters involved in metabolism and transport of immunosuppressive agents

Immunosuppressive agents	Metabolizing enzyme	Transporter protein
Cyclosporine A	CYP3A4	P-glycoprotein
Tacrolimus	CYP3A4	P-glycoprotein
Sirolimus	CYP3A4	P-glycoprotein
Mycophenolic acid	UGT1A9, UGT1A1, UGT1A6	MRP2

It is thus evident from the various literature reports that alterations in the metabolizing enzymes (CYPs and UGTs) and/or drug transporters (P-glycoprotein, MRP2) can significantly alter the pharmacokinetic parameters of immunosuppressive agents. Inhibition of drug metabolizing enzymes and /or drug transporters by endogenous and/or exogenous compounds

will result in increased levels of immunosuppressive agents leading to toxic effects. Conversely, their induction will result in decreased overall body exposure to the drug causing organ rejection.

Thus, it is important to characterize the effect of various endogenous as well as exogenous chemicals on drug metabolizing enzymes and transporters that are involved in the metabolism and transport of various immunosuppressive agents.

In this dissertation, we will discuss the effect of endogenous chemicals such as pro-inflammatory cytokines and exogenous drugs such as HIV-protease inhibitors on hepatic phase I and phase II metabolizing enzymes and drug transporters associated with metabolism and transport of immunosuppressants using human hepatocyte culture system.

1.7. Use of human hepatocytes to study hepatic drug metabolism and drug transport

There are various *in vitro* or *in vivo* in animals or human models to study the hepatic drug metabolism and transporters (Table 7). As the complexity of the system increases, the similarity to the *in vivo* situation also increases. Human studies are believed to be a more accurate indicator of drug metabolism, as they comprise all of the biological process that will interact with a compound in one contained system. However, due to ethical considerations it is difficult to carry out human studies routinely. *In vivo* animal studies are also considered more accurate indicator of drug metabolism, but have the same limitation because of ethical issues and the cost involved. Liver slices and whole perfused livers have the advantages of maintaining the *in vivo* liver architecture, yet, similar to suspended hepatocyte cultures; have the disadvantage of viability for only 4-6 hours and the results show poor reproducibility.

Human hepatocytes are a more releavant *in vitro* system due to their strong resemblance to *in vivo* human liver. Primary cultures of human hepatocytes (PCHH) are viable for up to 2 weeks, or one month if placed in a three-dimensional culture, and retain all cofactors and

cosubstrates necessary for phase I and phase II processes, making them a versatile *in vitro* system to study induction and inhibition of drug metabolism (Gebhardt et al., 2003). They are also valuable in characterizing the metabolic profile of a drug, studying the interspecies differences in drug metabolism, assessing drug-drug or drug-endogenous compound interactions and predicting the *in vivo* behavior of a drug.

Table 7. Systems used to study hepatic drug metabolism and transporters

System	Complexity	Resemblance	Ease of use
Subcellular fractions Supersomes Microsomes Cytosol			
Human hepatocyte cultures Suspended cultures Tumor derived cell lines Cryopreserved hepatocytes Primary hepatocytes 3D cultures			
Liver slices			
Whole liver perfusion			
***In vivo* animal model**			
Human			

As the use of PCHH has advanced, modified culturing techniques have enabled the examination of other processes involved in drug metabolism, namely the uptake and efflux of drugs and their metabolites by hepatic drug transporters. The loss of tight junctions secondary to the hepatocyte isolation procedure results in a loss of cellular polarity, or depolarization, and results in changes in hepatically expressed genes. Normal monolayered PCHH show reduced albumin secretion over time, increased levels of alpha-fetoprotein, a protein that is associated with depolarization and dedifferentiation of hepatocytes, dephosphorylation of cell surface receptors responsive to growth factors and, in the case of rat hepatocytes, a rapid loss of drug metabolizing activity and MDR1 expression (Luttringer et al., 2002; Richert et al., 2002; Boess et al., 2003). The application of extracellular 3D matrix prevents the loss in albumin synthesis, leads to the phosphorylation of hepatocyte growth factor receptors and results in cuboidal, polar hepatocyte structure. This has been shown to result in relocalization of MDR1 in hepatic canalicular membrane (Sidhu and Omiecinski, 1995; Kudryavtseva and Engelhardt, 2003; Engl et al., 2004). Hepatocytes in 3D culture have been utilized to document the effects of a variety of compounds on MDR, NTCP, MRP2 and BSEP expression and activity (Liu et al., 1999; Luttringer et al., 2002; Kostrubsky et al., 2003; Hoffmaster et al., 2004).

1.8. Effect of inflammation and HIV protease inhibitors on hepatic drug metabolizing enzymes and transporters

1.8.1. Central hypothesis

Variation in the expression of nuclear receptors, PXR and CAR will alter the expression and activity of enzymes and transporters involved in the metabolism and transport of immunosuppressive drugs. The expression of PXR and CAR can be modulated by endogenous mediators such as cytokines and exogenous compounds such as HIV-protease inhibitors.

Cultured human hepatocytes are a valuable tool to evaluate alterations in the expression and activity of drug metabolizing enzymes and transporters.

Hypothesis 1

We hypothesize that HIV protease inhibitors have a dual effect on CYP3A4 enzyme, with different potencies for different protease inhibitors. HIV-protease inhibitors activate PXR, and increase CYP mRNA and protein. However, as they are substrates for CYP3A4, despite an increase in CYP3A4 mRNA and protein, the activity of CYP3A4 enzyme measured by a probe substrate will be decreased in the presence of HIV-protease inhibitors. Combination of protease inhibitors with rifampicin, which is a prototypical inducer of CYP3A4, will result in further increased CYP3A4 expression and activity. Removal of protease inhibitors from the vicinity of the enzyme-binding site after chronic exposure will result in the complete reversal of the effects seen on CYP3A4 expression and activity.

In these studies, we have used primary cultures of human hepatocytes to evaluate the concentration-dependent effect of ritonavir, indinavir and amprenavir on CYP3A mRNA expression, protein content and enzyme activity. The effect of removal of ritonavir and amprenavir, after initial chronic exposure on the recovery of CYP3A4 expression and activity was also studied. The effect of HIV- protease inhibitors in combination with rifampicin was also

evaluated **(Chapter 3).** CYP3A4 enzyme was selected for this study as it is involved in the metabolism of several immunosuppressive drugs such as cyclosporine, tacrolimus and sirolimus.

Hypothesis 2

We hypothesize that HIV protease inhibitors will 'indirectly' modulate the expression and activity of UGT1A1 enzyme, by affecting the expression of regulatory pathways such as the expression of PXR and CAR. Primary cultures of human hepatocytes were used to characterize the effect of HIV protease inhibitors namely ritonavir, indinavir and amprenavir on the expression and activity of UGT1A1. The enzyme UGT1A1 was selected in this study as it is involved in the metabolism of mycophenolic acid (an immunosuppressive drug) and bilirubin (a byproduct of liver metabolism) **(Chapter 4).**

Hypothesis 3

We hypothesize that HIV protease inhibitors will alter the expression of hepatic drug transporters by modulating the expression of PXR and CAR, which are involved in their regulation. Primary cultures of human hepatocytes were used to determine the effect of increasing concentrations of HIV protease inhibitors such as ritonavir, indinavir and amprenavir on the expression of canalicular drug transporters namely P-glycoprotein, MRP2, MRP6 and BSEP **(Chapter 5)**. BSEP was selected for this study as it is involved in canalicular transport of bile salts that are released in the liver.

Hypothesis 4

We hypothesize that in human hepatocytes, cytokines will downregulate the expression and activity of hepatic efflux transporters and this suppression will be due to alterations in the expression of transcriptional factors, PXR and/or CAR. The effect of increasing concentrations

of IL-1β, IL-2, IL-6 and TNFα on the expression and activity of bile salt export pump (BSEP) were determined using human hepatocyte cultures. **(Chapter 6)**

Hypothesis 5

We hypothesize that pro-inflammatory cytokines will downregulate expression and activity of hepatic UGT1A1 enzyme and this suppression will be due to alterations in the expression of transcription factors, PXR and/or CAR. The effect of increasing concentrations of cytokines such as IL-1β, IL-2, IL-6 and TNFα on the expression and activity of UGT1A1 were studied using primary cultures of human hepatocytes. **(Chapter 7)**

2. Materials and Methods

2.1. Chemicals

A modified Williams E culture medium (HMM), medium supplements, dexamethasone and insulin, were obtained from BioWhittaker (Walkersville, MD). Penicillin G/streptomycin was obtained from Gibco Laboratories (Grand Island, NY). Rifampicin (RIF), phenobarbital (PB) and testosterone (TE) were obtained from Sigma (St. Louis, MO). 6β-Hydroxytestosterone was obtained from Steraloids (Wilton, NH). Various protease inhibitors were obtained for research purpose from the following sources; amprenavir, ritonavir (Abbott), indinavir (Merck), nelfinavir (GMP), lopinavir (Abbott) and saquinavir (Roche). All four cytokines namely IL-1, IL-2, IL-6 and TNFα were obtained from Sigma. Falcon 6-well culture plates were obtained from Becton Labware (Franklin Lakes, NJ). BSA standard for total protein measurement by Lowri was obtained from Sigma. Nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) color developing reagent and alkaline phosphatase-conjugated anti-rabbit and anti-goat antibodies were purchased from Bio-Rad (Richmond, CA). Baculovirus-expressed CYP3A4 and UGT1A1 were obtained from BD Gentest (Woburn, MA). Antibodies used to detect CYP3A4 (# 458334) and UGT1A1 (# 456411) were purchased from BD Genest (Woburn, MA). Reagents for reverse transcription were purchased from Promega (Madison, WI). Forward and reverse primers CYP3A4, UGT1A1, BSEP and β-actin were synthesized by Applied Biosystems. Primers for all transporters and Taqman probe were purchased from Applied Biosystems. The assay ids are as follows: BSEP (Hs00184824_m1), MRP6 (Hs00184566_m1), PXR (Hs0018243666_m1), CAR (Hs00231959_m1). Primer sequence for each gene is found in Table 8. All solvents and other chemicals used were of HPLC grade or the highest purity available.

Table 8. Real-Time PCR primers for genes detected by SYBR Green

Gene	Name	Forward Primer (5' to 3')	Detection
CYP3A4	Forward	CTT CAT CCA ATG GAC TGC ATA AAT	SYBR Green
	Reverse	TCC CAA GTA TAA CAC TCT ACA CAG ACA A	
UGT1A1	Forward	TGT TGG TGG AAT CAA CTG CCT	SYBR Green
	Reverse	TGC CCA AAG CAT CAG CAA TT	
β-actin	Forward	AGG CAT CCT CAC CCT GAA GTA	SYBR Green
	Reverse	CAC ACG CAG CTC ATT GTA GA	
MRP2	Forward	TGCAGCCTCCATAACCATGAG	SYBR Green
	Reverse	GATGCCTGCCATTGGACCTA	

2.2. Hepatocyte isolation

Primary cultures of human hepatocytes (PCHH) were prepared by a three-step collagenase perfusion technique (Strom et al., 1996). Viability of cells was determined by the trypan blue exclusion method and cells were used only when the viability was at least 65%. Briefly, equal volumes of trypan blue (0.4%) and cell suspension were mixed and a portion of this suspension was then placed on a hemocytometer. The cells were observed under a light microscopy and the numbers of live and dead cells (stained blue), were counted in two fields. Concentration of cells (number of cells / ml) was determined using the following formula: Live cells in two fields x 10,000 = # of cells/ml. Cells were diluted to final volume of 1 x 10^6 cells per ml.

Hepatocytes were plated on Falcon 6-well culture plates (1.5 x 10^6 cells), previously coated with rat tail collagen in HMM supplemented with 0.1 μM insulin, 0.1 μM dexamethasone, 0.05% streptomycin, 0.05% penicillin, 0.05% amphotericin B and 10% bovine

calf serum. After allowing the cells to attach for 4 to 6 hours, medium was replaced with serum-free medium containing all of the supplements described above. Cells were maintained in culture at 37°C in an atmosphere containing 5% CO_2 and 95% air.

After 24 hours in culture, unattached cells were removed by gentle agitation and the medium was changed. For transporter studies, cells were overlayed with Matrigel™ (0.233 mg/mL) at this time. The medium was changed every 24 hours and the hepatocytes were maintained in culture between 5 and 9 days depending on the experimental design.

2.3. General hepatocyte treatment

Briefly, hepatocytes were maintained in culture in the presence of the chemical under study or vehicle control (DMSO 0.1% or MeOH 0.1 %). On the day of the experiment, cells were washed with HMM devoid of insulin, dexamethasone, antibiotics and antifungal drugs. It is assumed that this one-hour period is sufficient to remove residual chemical from the enzyme active site. Following this period, media containing the appropriate probe substrate was applied to the cells with media sampled at the appropriate time points. Table 9 summarizes the enzymes studied with the respective probe substrates, concentrations used and sampling times. A variety of variations on the traditional methods used to assess drug metabolizing enzyme activity are discussed in the subsequent chapters.

2.4. Analytical Methods

Only the analytical methods used to assess enzyme activity that are used in multiple chapters are described below.

2.4.1. HPLC measurement of CYP3A4 activity

The concentration of 6β-hydroxytestosterone in the medium was measured by HPLC as previously described, with the following modifications (Kostrubsky et al., 1999). One hundred microliters of medium were diluted with an equal volume methanol and centrifuged at 13,000 r.p.m. for 5 minutes. One hundred microliters of this solution was injected onto a LiChrospher 100 RP-18 column (4.6 x 250 mm, 5 μm). 6β-hydroxytestosterone was eluted with a mobile phase of methanol/water (60:40, v/v) at a flow rate of 1.2 ml/min and the eluents were monitored at 242 nm. The concentration of the metabolite was quantitated by comparing the peak areas in samples to a standard curve containing known amount of the metabolite.

2.4.2. HPLC measurement of UGT1A1 activity

The concentration of estradiol-3-glucuronide in the cell lysate was measured by HPLC as previously described, with the following modifications (Alkharfy and Frye, 2002). Medium aliquots were centrifuged at 12,000 g for 5 min to remove large particulates. A 50 μL aliquote of lysate was injected directly onto a Alltima C18 column (250 x 4.6 mm, 5 μm; Waters Corp., Milford, MA). Estradiol-3-glucuronide was eluted at a flow rate of 1 ml/min using with a mobile phase of acetonitrile/50mM ammonium phosphate buffer (pH 3) (35/65, v/v). The metabolite was measured using a fluorescence detector (Waters 474) with excitation and emission wavelengths of 210 and 300 nm, respectively.

Table 9. Probe substrates to study enzyme and transporter activity in human hepatocyte cultures

Enzyme	Probe substrate	Probe Conc. (µM)	Metabolite	Standard inducer	Inducer Conc. (µM)	Incubation Time[a]	Analysis
CYP3A	Testosterone	250	6β(OH) testosterone	Rifampin	10	30 min	HPLC
UGT1A1	Estradiol	250	Estradiol-3-glucuronide	Phenobarbitol	2000	60 min	HPLC
BSEP	3[H] taurocholate	1	N/A	N/A	N/A	20 min	Scintillation counter

[a]In linear portion of concentration versus incubation time profile.

2.4.3. Determination of total protein

After sampling of the medium for metabolite measurements, the remainder of medium (0.5 mL) was aspirated from each well. Cells were then harvested in 150 µL of phosphate buffer and stored at -80°C for protein determination by the method of Lowry (Lowry O, 1951). Briefly, the proteins were dissolved in SDS/sodium hydroxide, then 1% sodium tartarate and 1% copper sulfate were added, followed by the addition of Folin's reagent. The tubes were mixed gently and the color was allowed to develop for 45 min. At the end of 45 minutes, 200 µL aliquotes were transferred to 96-well plates and the absorbance was measured at 490 nm. The concentration of the protein was calculated using bovine serum albumin as a protein standard.

2.4.4. Measurement of immunodetectable protein

Immunochemical detection of CYP3A and UGT1A1 was performed as previously described (Kostrubsky et al., 1995). Briefly, 20 µg of pooled total cellular protein was loaded onto a polyacrylamide gel and subjected to SDS-PAGE at 80 volts for 90 minutes. Proteins were transferred onto a nitrocellulose membrane for 3 hours and blocked overnight at 4°C. Membranes were washed and incubated with the appropriate anti-CYP or anti-UGT1A1 antibody (all diluted 1:1000) for 2 hours at room temp on a rocker table. The membrane was again washed and then incubated with a horseradish perxodase labeled secondary antibody diluted 1:10,000 for 1 hour at room temperature on a rocker table. Blots were developed using a Nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate developing reagent. Relative amounts of proteins were assessed by the intensity of immunoblot staining carried out by densitometry (ImageJ, v1.34, http://reb.info.nih.gov/ij). All densitometry results were normalized to β-actin and then to appropriate vehicle control value.

2.4.5. Measurement of mRNA expression of metabolizing enzymes and transporter

Total RNA was extracted from 1 x 10^6 cells plated on 6-well plates using 1 mL Trizol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. RNA was quantified spectrophotometrically and subjected to agarose gel electrophoresis to assess the integrity of RNA. Following treatment with RNase-free DNase (Promega, Madison, WI), 2 μg of RNA was mixed with 0.5 μg of Random Hexamers (Promega) heated to 70°C for 5 minutes then cooled to 4°C. A reaction mixture containing 200 U MMLV-Reverse transcriptase, 1 mM dNTPs and 25 U RNasin (Promega) was added to the previous mixture and incubated at 37°C for 60 minutes. The resulting cDNA was diluted 10-fold and stored at -20°C.

2.4.6. Real-Time PCR

Primers for β-actin and CYP3A4 (Bowen et al., 2000) were designed using PrimerExpress 1.0 (Applied Biosystems, Foster City, CA). Sequences of primer for UGT1A1 was obtained from personal correspondence with Dr. Federico Innocenti (University of Chicago, Chicago, IL PCR was performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using 5 μl of cDNA, 200 pM of forward and reverse primers (SYBR green technology) or 1.25 μl Assays on Demand-Mix (TaqMan® technology) and 12.5 μl PCR Master Mix (Applied Biosystems) for a total volume of 25 μl. PCR conditions were 50°C for 2 min, 95°C for 10 min, followed by 50 cycles with 15 sec at 95°C and 1 min at 60°C. The relative cDNA content was determined from standard curves constructed from serial by diluted cDNA and normalized to β-actin in each sample.

3. Effect of HIV protease inhibitors on hepatic phase I drug metabolizing enzyme in human hepatocytes

3.1. Abbreviations

6β(OH)TE	6β(OH) testosterone
APV	amprenavir
CYP	cytochrome P450
CYP3A4	cytochrome P4503A4
HMM	hepatocyte maintenance medium
IDV	indinavir
LPV	lopinavir
NFV	nelfinavir
PCHH	primary cultures of human hepatocytes
RIF	rifampicin
RTV	ritonavir
SQV	saquinavir
TE	testosterone

3.2. Abstract

Aims: Clinically significant drug-drug interactions are observed in HIV patients undergoing transplantation where immunosuppressive agents are co-administered with HIV-protease inhibitors. CYP3A4 being the primary enzyme involved in the metabolism of immunosuppressive agents, these interactions are thought to be due to alterations in CYP3A4 enzyme by HIV-protease inhibitors. The objective of the study was to use primary cultures of human hepatocytes (PCHH) to study the concentration-dependent effect of HIV-protease inhibitors namely ritonavir, indinavir and amprenavir on CYP3A4 expression and activity. We also studied the possible synergistic interaction of HIV-protease inhibitors and a prototype PXR activator rifampicin, a potent CYP3A4 inducer. Furthermore, the effect of removal of ritonavir and amprenavir, after initial chronic exposure on the recovery of CYP3A4 expression and activity was also studied to understand the time course of recovery of enzyme expression and activity.

Methods: To determine the effect of various concentrations, PCHH were exposed to ritonavir (RTV), indinavir (IDV) and amprenavir (APV) (0-10 μM) for 72 hours. In studies involving interactions, cells were exposed to the combination of protease inhibitor (10 μM) + rifampicin (RIF) (10 μM) for 72 hours. The effect of chronic treatment and subsequent washout of drug was studied by treating the cells with either RTV or APV (1 and 10 μM) for 72 hours followed by 72 hours washout period. CYP3A4 mRNA expression was determined by quantitative Real-time PCR, while protein content was evaluated using western blotting. CYP3A4 activity was assessed using testosterone (TE) as a probe substrate.

Results: Protease inhibitors inhibited CYP3A4 activity in the decreasing order of inhibition potency; ritonavir > indinavir > amprenavir, with ritonavir being the most potent inhibitor. Protease inhibitors are shown to interact with RIF and maximal interaction was observed for the combination of RTV + RIF, where RTV completely masked the CYP3A4 inducing ability of RIF. In the recovery study, increased mRNA expression and activity, after APV treatment, completely recovered to the baseline value during a 72 hours washout. On the other hand, recovery of CYP3A4 activity after RTV (10 μM) was incomplete after 72 hours and was predicted to reach the baseline value after 244 hour of drug washout period.

Conclusions: This study has demonstrated the concentration dependent effect of individual protease inhibitors on CYP3A4 expression and activity in a human hepatocyte system. Secondly, the interaction potential of these protease inhibitors with currently known CYP3A4 substrates and inducers has also been shown. Additionally, the reversible nature of CYP3A4 inhibition or induction by protease inhibitors after treatment and removal has been documented. The calculation of kinetic parameters such as $t_{1/2}$ and recovery time for CYP3A4 expression and activity after drug discontinuation can help to better understand the duration of the effect of HIV-protease inhibitors on CYP3A4 expression and activity.

3.3. Introduction

Historically, human immunodeficiency virus (HIV) infection has been considered as an absolute contraindication to transplantation because of the high mortality rate of opportunistic infection associated with HIV infection and anti-rejection immunosuppression. However, liver transplantation is now considered as a treatment of choice in HIV-patients with end stage liver disease, because of improvement in immune function and survival made possible by the introduction of highly active antiretroviral therapy (HAART) (Ragni et al., 2003; Neff et al., 2004). Currently there are eight protease inhibitors approved by FDA for treatment of HIV infection. Ritonavir, lopinavir, indinavir, nelfinavir, amprenavir and saquinavir, are some of the most commonly used HIV protease inhibitors most either alone or in combination (http://www.fda.gov/oashi/aids/virals.html)

Clinically significant pharmacokinetic interactions between immunosuppressive agents and HIV- protease inhibitors have been observed in HIV positive patients undergoing liver transplantation, requiring dosing adjustments of the immunosuppressive drugs (Sheikh et al., 1999; Schvarcz et al., 2000; Jain et al., 2002a; Jain et al., 2002b; Jain et al., 2003; Schonder et al., 2003). Inhibition of CYP3A4, the principal enzyme involved in the metabolism of most of the immunosuppressive agents and P-glycoprotein involved in efflux transport of immunosuppressive agents, by HIV-protease inhibitors is considered to be the most likely mechanism responsible for these drug interactions.

In vitro metabolism studies have shown that all the HIV protease inhibitors are predominantly metabolized by CYP3A enzymes with a minor contribution from CYP2D6 and CYP2C9 (Kumar et al., 1996; Chiba et al., 1997; Fitzsimmons and Collins, 1997).

HIV protease inhibitors have complex effects on the CYP3A family of enzymes. Studies in rat or human microsomes and expressed enzyme systems have shown that HIV- protease inhibitors inhibit CYP3A4 enzyme to a varying degree. Ritonavir is by far the most potent inhibitor, followed by indinavir, nelfinavir, amprenavir and saquinavir in decreasing order of potency (Eagling et al., 1997; Decker et al., 1998; Iribarne et al., 1998; Koudriakova et al., 1998). Conversely, ritonavir and nelfinavir have also shown to induce CYP3A4 in studies carried out in rats and in human colon carcinoma cell lines (Greenblatt et al., 2000; Perloff et al., 2000; Venkatakrishnan et al., 2000). This induction phenomenon is of particular interest since all these protease inhibitors are used over an extended period of time in clinical practice. The induction of CYP3A4 by protease inhibitors may lead to decreased plasma drug levels of co-administered drugs that are substrates of CYP3A4, thus leading to loss of their therapeutic efficacy.

It is known that CYP3A4 is transcriptionally regulated by the nuclear orphan receptor pregnane X receptor (PXR). After ligand binding in the cytosol, PXR translocates to nucleus where is heterodimerizes with retinoid X receptor (RXR), and then binds to the *CYP3A4* promotor, resulting in the increased CYP3A4 mRNA expression. *In vitro* studies have shown that ritonavir is a potent activator of PXR (Luo et al., 2002).

Since most of the studies documenting the inhibitory effect of protease inhibitors on CYP3A4 have been carried out either in microsomes, expressed enzyme systems or colon carcinoma cell lines, the potential for the induction of CYP3A4 enzyme after exposure to protease inhibitors has not been evaluated. Also, there is no systematic study showing the effect of variable concentrations of these HIV protease inhibitors on the expression and activity of CYP3A4 and on expression of nuclear receptors believed to be responsible for modulating activity of drug metabolizing enzymes.

We hypothesized that HIV protease inhibitors have a dual effect of inhibition and induction on CYP3A4 enzyme with different potencies for different protease inhibitors. We hypothesized that HIV-protease inhibitors will activate PXR; increase CYP3A4 mRNA expression and protein. However, as they are substrates for CYP3A4, in spite of an increase in CYP3A protein, the activity of CYP3A4 as measured with another substrate will be decreased. Combination of protease inhibitors with rifampicin, which also activates PXR, will result in further increased CYP3A4 expression and activity. Removal of protease inhibitors from the vicinity of enzyme binding site after chronic exposure will result in the complete reversal of the effects seen on CYP3A4 expression and activity over time.

We used primary cultures of human hepatocytes where we can simultaneously measure mRNA, protein and activity to study the concentration-dependent effect of ritonavir, indinavir and amprenavir on CYP3A4 mRNA expression, protein content and enzyme activity. We also evaluated the possible drug interaction effect on CYP3A4 when HIV protease inhibitors were combined with rifampicin. The effect of removal of ritonavir and amprenavir, after chronic exposure on the recovery of CYP3A4 expression and activity was also studied.

3.4. Methods

3.4.1. Evaluation of the cytotoxicity of HIV protease inhibitors to human hepatocytes

Hepatocytes were exposed to HIV protease inhibitors RTV, IDV, and APV (0–50 μM) for 72 h. At the end of the treatment period, the media was aspirated and 10% v/v of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to HMM followed by 30 minutes of incubation. At 30 min, the medium was aspirated and cells were washed with HMM. Isopropanol (equal volume as the medium) was then added and shaken

gently for 2-3 min. Two hundred microliters of this solution was then transferred to a 96-well plate, and the absorbance was measured at 570 nm.

3.4.2. Hepatocyte treatment protocol to study the effect of different HIV protease inhibitors on CYP3A

To determine the effect of increasing concentrations of protease inhibitors on CYP3A4 enzyme expression and activity, twenty-four hours after plating, cells were exposed to RTV, IDV and APV (0-10 μM) for 72 hours. To study the interaction of various HIV protease inhibitors (10 μM) with rifampicin (10 μM), cells were exposed to appropriate combination of these chemicals for 72 hours. To evaluate the effect of removal of ritonavir and amprenavir, on the CYP3A4 enzyme expression and activity, hepatocytes were first treated with RTV and APV (1 and 10 μM) for 72 hours. The medium was then replaced with fresh medium every 24 hours for next 72 hours.

On the day of the study, cells were washed with 1.5 ml of fresh medium for 1 h and then incubated in 1.5 ml of medium containing 250μM testosterone for an additional 30 minutes. At the end of that time, 1 mL of medium was sampled and stored at -80°C for 6β(OH) testosterone (6β(OH) TE) determination by HPLC. The remaining media was aspirated, and the cells were harvested in phosphate buffer (0.1 M, pH 7.4) and stored at -80°C for protein determination and detection of immunoreactive CYP protein (Lowry O, 1951). The relative amounts of proteins were measured by the intensity of immunoblot staining carried out by densitometry (ImageJ, v1.34, http://reb.info.nih.gov/ij).

Cells were also harvested for mRNA by adding 1 mL of Trizol reagent to each well of a 6-well plate. The RNA samples were stored at -20°C for Real Time PCR analysis. Primers for CYP3A4, PXR, CAR, β-actin and the PCR procedure have been described in Chapter 2

(Materials and Methods, Table 8). The relative cDNA content was determined from standard curves constructed from serially diluted human cDNA samples. The mRNA expression for all genes was normalized to β-actin in each sample and expressed as fold change over control treatment.

3.4.3. Data analysis

The data were analyzed using a one-way analysis of variance with a post hoc Tukey's multiple comparison procedure. p value of ≤ 0.05 was considered statistically significant. All statistical analysis was performed using PRISM software version 4.0 (GraphPad Software, Inc., San Diego, CA, USA).

3.5. Results

Hepatocytes from a total of 16 liver donors were used to conduct the experiments outlined in this Chapter. Their relevant demographics, drug history and cell viability information is cited in Table 10.

3.5.1. Effects of chronic exposure of hepatocytes to HIV protease inhibitors on MTT reduction

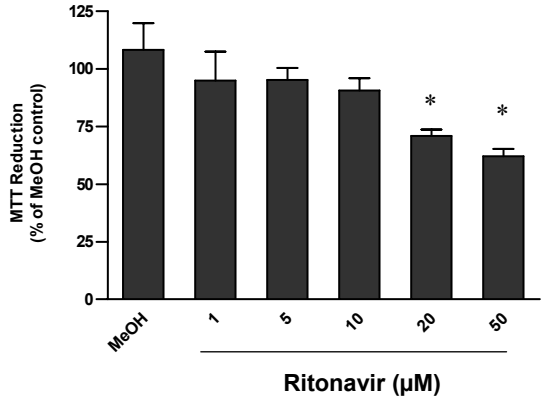
Chronic exposure (72 hours) of human hepatocytes to RTV, IDV and APV at concentrations ≥ 20 μM resulted in significant cellular toxicity as compared to the untreated cells (Figure 4). Thus, for all the further experiments, protease inhibitors at concentrations ≤ 10 μM were used.

Table 10. Donor information for human hepatocyte preparations used in Chapter 3

Donor HH #	Age	Sex[a]	Race[b]	Cause of death[c]	Drug History	Viability	Percoll separation
1117	68y	F	C	ICH/ Stroke	Labetolol, Verapamil, Clonidine	82	No
1118	73y	F	C	Head trauma	Atenolol, Imipramine	80	No
1129	70y	F	C	-	None reported	80	No
1135	53y	M	C	-	None reported	88	Yes
1184	66y	F	C	-	None reported	87	Yes
1200	53y	F	C	CVA/ Stroke	Dopamine	65	Yes
1201	69y	M	C	CA/ Anoxia	Epinephrine, Ampicillin	81	No
1205	45y	M	H	CVA/ Stroke	-	66	No
1209	30y	F	C	CVA/ Stroke	Heparin, Ampicillin, Gentamycin, Morphine	66	Yes
1210	35y	F	C	Head trauma	Dopamine, Vasopressin, hydrocortisone	83	Yes
1218	50y	F	C	CA/ Anoxia	-	85	No
1222	68y	F	C	-	-	93	No
1227	34y	M	C	CVA/ Stroke	-	74	Yes
1235	65y	M	C	-	-	78	No
1247	3y	M	-	-	-	71	No
1249	59y	M	H	Stroke	-	80	No

[a]M, male; F, female; [b]C, Caucasian; H, Hispanic; [c] CA, cardiac arrest; ICH, intra cranial hemorrahage

A.



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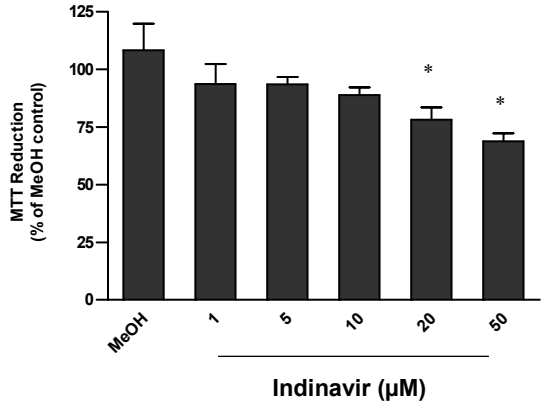

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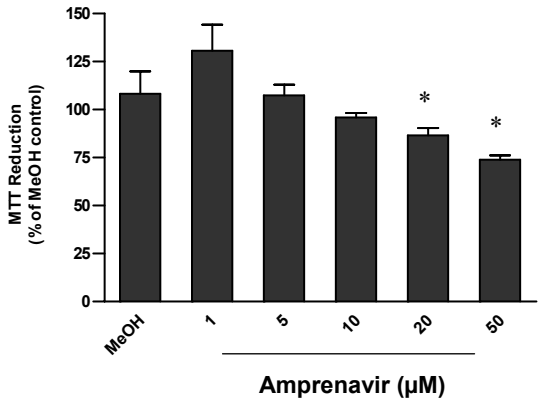


Figure 4. Effect of HIV protease inhibitors on MTT reduction

Hepatocytes were treated with A) RTV, B) IDV and C) APV (0–50μM) for 72 hours. MTT reduction was then measured. The figure shows the mean of triplicate treatments from two donors. The means are expressed as a percentage of the value obtained with vehicle treated cells, with the S.D. indicated by the vertical bars. *, significantly different from vehicle treated cells, $p \leq 0.05$.

3.5.2. Effects of HIV protease inhibitors on CYP3A4 expression and activity

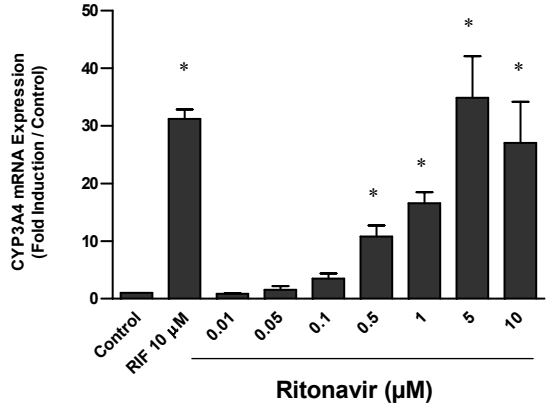
To determine the effect of increasing concentrations of HIV protease inhibitors on CYP3A4 enzyme expression and activity, cells were exposed to RTV, IDV or APV (0–10 μM) for 72 hours.

Treatment with the prototypical inducer RIF (10 μM) resulted in 31.2 ± 1.5- fold increase in the mRNA expression of CYP3A and 6.3 ± 1.2-fold increase in CYP3A4 protein content. After RIF (10 μM), the formation rate of 6β(OH) TE was increased 8.5 ± 2.5- fold compared to vehicle control.

3.5.2.1. Effects of ritonavir on CYP3A4 expression and activity

Treatment with RTV significantly increased the CYP3A4 mRNA expression at concentrations ≥ 0.5 μM (EC_{50} = 0.8954μM). This increase in CYP3A4 mRNA expression was 10 ± 1.9 fold and 27 ± 7.1 fold at concentrations 0.5 μM and 10 μM, respectively (Figure 5A). Ritonavir at concentrations ≥ 1 μM showed a 4-fold increase in the CYP3A4 protein content (Figure 5B). The increase in CYP3A4 mRNA and protein expression, however, was not associated with increased CYP3A4 enzyme activity. CYP3A4 mediated testosterone metabolism was significantly inhibited at all the concentrations of RTV used (IC_{50} = 0.01907 μM) (Figure 5C). At the lowest concentrations of RTV (0.01 μM), 6β(OH) TE formation rate was inhibited to 61.2 ± 12.5 %, while at the highest concentration (10 μM), the 6β(OH) TE formation rate was 2.0 ± 2.8 %, of control activity.

A.



EC_{50} = 0.8954 μM

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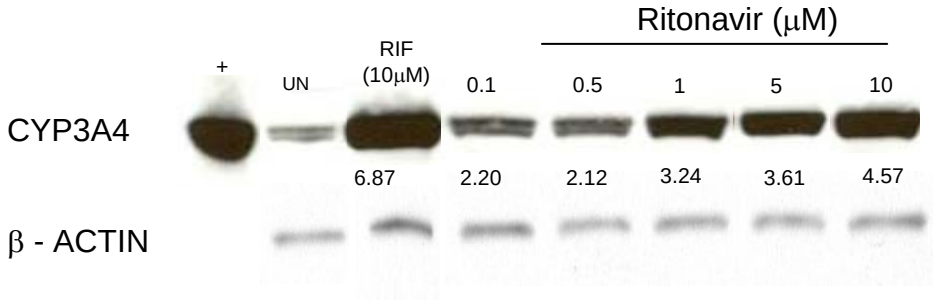

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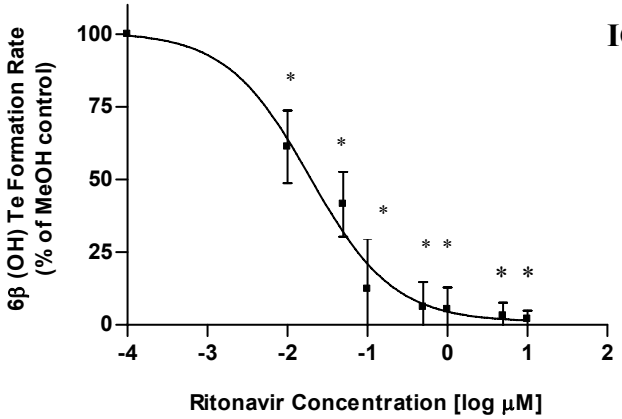


IC_{50} = 0.01907 μM

Figure 5. Effect of ritonavir on CYP3A4 mRNA expression, protein content and activity

Hepatocytes were exposed to RTV (0-10μM) for 72 hours and CYP3A4 A) mRNA expression, B) protein content and C) activity were determined. The figure shows the mean of values obtained from three livers, with the S.D. indicated by the vertical bars. All mRNA and protein values are normalized to β-actin expression. *, significantly different from untreated control cells, $p \leq 0.05$.

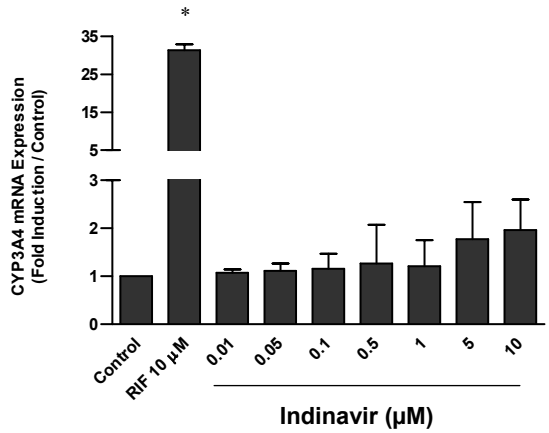
3.5.2.2. Effects of indinavir on CYP3A4 expression and activity

Exposure of cells to IDV (0-10 μM) did not result in any significant differences in CYP3A4 mRNA expression (Figure 6A). Indinavir did not show any significant increase in CYP3A4 protein content (Figure 6B). Indinavir significantly inhibited the CYP3A4 mediated testosterone metabolism at concentrations ≥ 0.1 μM (IC_{50} =0.3297 μM). The maximum inhibitory effect of IDV was seen at 5 μM. At IDV 5 μM 6β(OH) TE formation rate was 44.6 ± 2.7 % of control treatment (Figure 6C).

3.5.2.3. Effects of amprenavir on CYP3A4 expression and activity

Hepatocyte treatment with APV (0-10μM) showed significant increase in CYP3A4 mRNA expression at concentrations ≥ 1 μM, with the maximal increase being 9.7 ± 1.2-fold compared to vehicle control (Figure 7A). This increase in mRNA expression was associated with a 4.34-fold increase in CYP3A4 protein content (Figure 7B). Amprenavir at concentrations ≥ 5μM showed a concentration dependent increase in CYP3A4 activity. At 10 μM APV the formation rate of 6β(OH) TE was 1.68 ± 0.2-fold compared to control (Figure 7C).

A.



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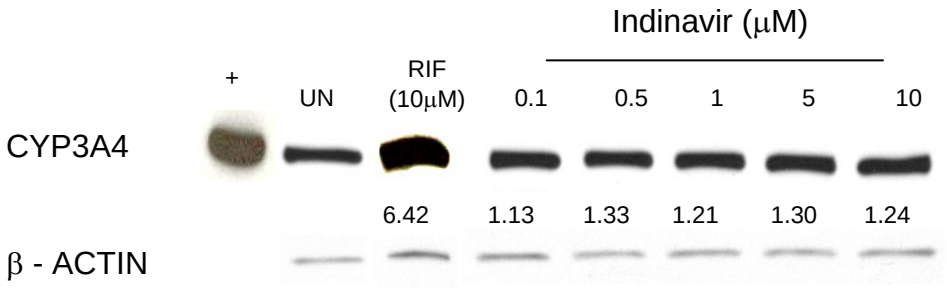

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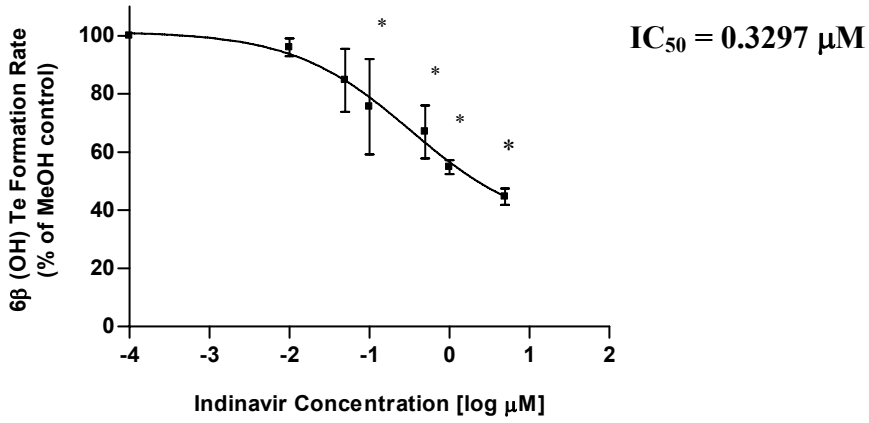


Figure 6. Effect of indinavir on CYP3A4 mRNA expression, protein content and activity

Hepatocytes were exposed to IDV (0-10µM) for 72 hours and CYP3A4 A) mRNA expression, B) protein content and C) activity were determined. The figure shows the mean of values obtained from three livers, with the S.D. indicated by the vertical bars. All mRNA and protein values are normalized to β-actin expression. *, significantly different from untreated control cells, $p \leq 0.05$.

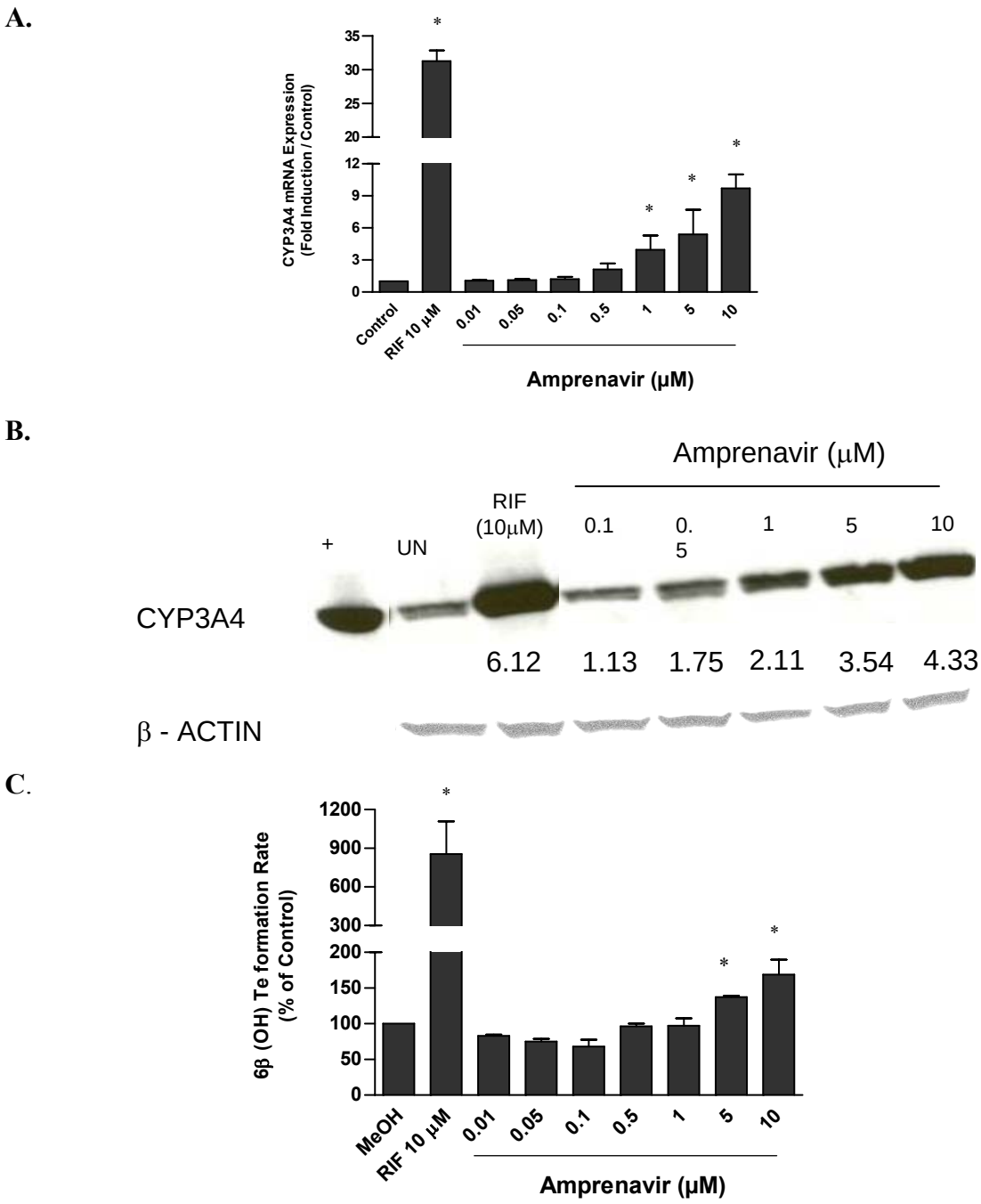


Figure 7. Effect of amprenavir on CYP3A4 mRNA expression, protein content and activity

Hepatocytes were exposed to APV (0 - 10 µM) for 72 hours and CYP3A4 A) mRNA expression, B) protein content and C) activity were determined. The figure shows the mean of values obtained from three livers, with the S.D. indicated by the vertical bars. All mRNA and protein values are normalized to β-actin expression. *, significantly different from untreated control cells, $p \leq 0.05$.

3.5.3. Effect of combination of HIV protease inhibitors with rifampicin on CYP3A4 expression and activity

To study the interaction between various HIV protease inhibitors and RIF, hepatocytes were exposed to the combination, protease inhibitor (10μM) + RIF (10μM) for 72 hours.

No cellular toxicity was observed in MTT reduction assay, after combining the protease inhibitor (10μM) with RIF (10μM) (Figure 8).

Treatment with RIF (10μM) showed 14.4 ± 6.4-fold increase in CYP3A4 mRNA expression, while the CYP3A4 protein content was increased 5.4-fold compared to control treatment. The formation rate of 6β(OH) TE was increased 19.1 ± 13.9-fold at RIF (10μM).

Figure 9 and Table 11 shows the effect on CYP3A4 expression and activity when RTV, IDV and APV were combined with RIF. After RTV (10μM) treatment, CYP3A4 mRNA expression was increased 17.3 ± 1.3-folds over control. The combination of RIF and RTV resulted in 23.3 ± 0.8 -fold increase in mRNA expression, which was significantly different than RIF (10 μM) alone (Figure 9A). Ritonavir (10μM) and RIF + RTV treatment resulted in 3-10 and 5-13-fold increase in CYP3A4 protein content, respectively (Figure 9B). But, RTV (10μM) as well as the combination of RTV + RIF resulted in complete inhibition of CYP3A4 mediated testosterone metabolism (Figure 9C).

Indinavir (10μM) showed a 4.6 ± 1.6-fold increase in mRNA expression, while RIF + IDV resulted in 16.7 ± 0.6-fold increase which was significantly different from the IDV (10μM) alone but was similar to the increase in CYP3A4 mRNA expression seen after RIF (10μM) alone. Indinavir alone did not result in significant increase in CYP3A4 protein content but IDV+RIF increased CYP3A4 protein 15.5-fold over control. The formation rate of 6β(OH) TE was 1.9 ± 1.1-fold and 15.7 ± 4.7-fold of control, after IDV (10 M) alone and RIF + IDV, respectively.

Amprenavir (10μM) alone showed a 8.6 ± 2.1-fold increase in mRNA expression, while RIF + APV resulted in 23.6 ± 5.2-fold increase which was similar to the increase in CYP3A4 mRNA expression seen after RIF (10μM) alone, but was significantly higher than APV treatment alone. The formation rate of 6β(OH) TE was 4.0 ± 0.5-fold and 14.2 ± 3.2-fold of control, after APV (10 μM) alone and RIF + APV, respectively. Figure 10 and Table 11 shows the effect on CYP3A4 expression and activity when LPV, NFV and SQV were combined with RIF.

The mRNA expression of CYP3A4 changed from 5.3 ± 0.3-fold (LPV alone) to 16 ± 2.2-fold in presence of RIF + LPV combination. Similarly, the increase in 6β(OH) TE formation rate was changed from 2.19 ± 0.1-fold (LPV alone) to 15.7 ± 4.7-fold in the presence of RIF + LPV combination.

Nelfinavir (10μM) alone showed an 8.8 ± 4.4-fold increase in mRNA expression, while RIF + NFV resulted in 27.2 ± 6.8-fold increase in CYP3A4 mRNA expression. The formation rate of 6β(OH) TE was 4.9 ± 3.4-fold and 15.5 ± 4.6-fold of control, after NFV (10μ M) alone and RIF + NFV, respectively. The increase in mRNA expression and activity was significantly different from that observed with NFV alone, but was not different from that observed after RIF treatment alone.

The mRNA expression of CYP3A4 changed from 3.9 ± 0.1-fold (SQV alone) to 32.9 ± 4.9-fold in presence of RIF + SQV combination. Similarly, the increase in 6β(OH) TE formation rate was changed from 2.2 ± 1.0-fold (LPV alone) to 19.4 ± 4.3-fold in the presence of RIF + SQV combination.

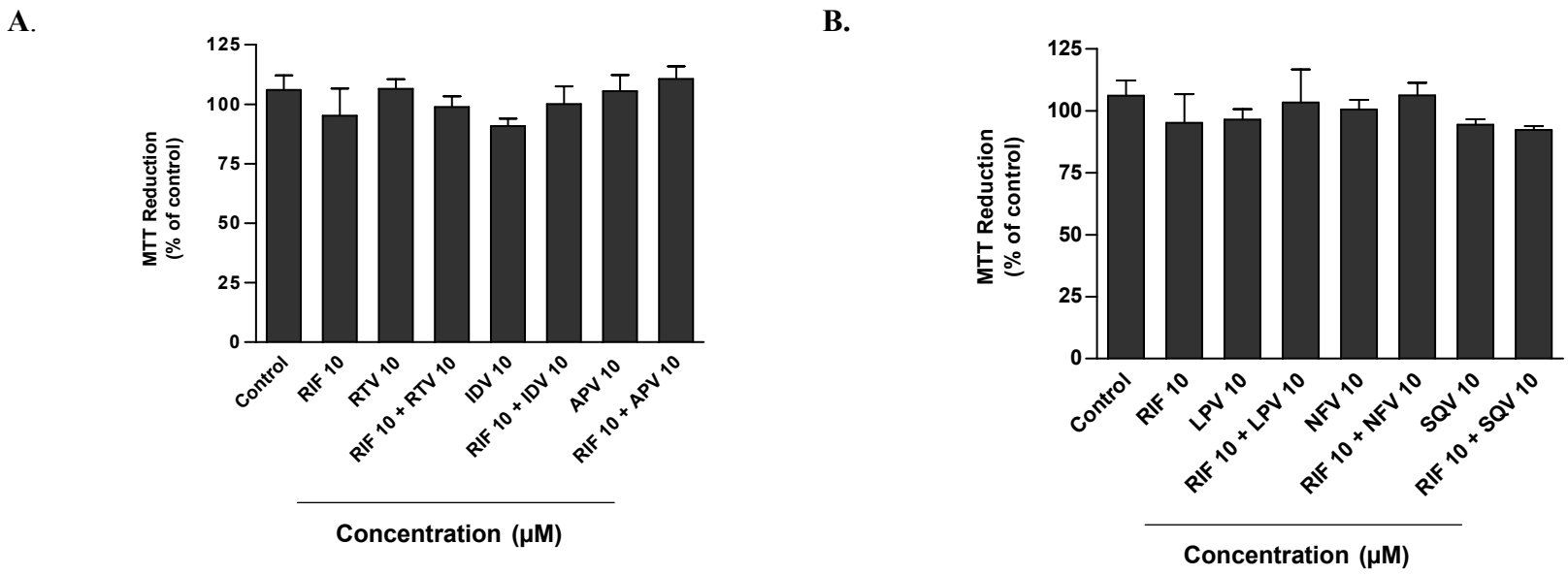


Figure 8. Effect of combination of HIV protease inhibitors and rifampicin on MTT reduction

Hepatocytes were treated with A) RTV, IDV and APV alone (10μM) or in combination with RIF (10 μM) B) LPV, NFV and SQV alone (10μM) or in combination with RIF (10 μM) for 72 hours. MTT reduction was then measured. The figure shows the mean of triplicate treatments from two donors. The means are expressed as a percentage of the value obtained with untreated control cells, with the S.D. indicated by the vertical bars. *, significantly different from vehicle control treated cells, $p \leq 0.05$.

Table 11. Effect of combination of protease inhibitor and rifampicin on CYP3A4 mRNA expression and activity

Treatment Conc (μM)	CYP3A4 mRNA expression (Fold / control)	6β (OH) Te formation rate (Fold / control)
RIF 10	14.4 ± 6.4	19.1 ± 13.9
RTV 10	17.3 ± 1.3	0.04 ± 0.04[a]
RTV 10 + RIF 10	23.3 ± 0.8[a]	0.03 ± 0.05[a]
RIF 10	14.4 ± 6.4	19.1 ± 13.9
IDV 10	4.6 ± 1.6[a]	1.9 ± 1.1
IDV 10 + RIF 10	16.7 ± 0.6[b]	15.7 ± 4.7[b]
RIF 10	14.4 ± 6.4	19.1 ± 13.9
APV 10	8.6 ± 2.1	4.0 ± 0.5
APV 10 + RIF 10	23.6 ± 5.2[b]	14.2 ± 3.2[b]
RIF 10	14.4 ± 6.4	19.1 ± 13.9
LPV 10	5.3 ± 0.3[a]	2.9 ± 0.1
LPV 10 + RIF 10	16 ± 2.2[b]	15.7 ± 4.7[b]
RIF 10	14.4 ± 6.4	19.1 ± 13.9
NFV 10	8.8 ± 4.4	4.9 ± 3.4
NFV 10 + RIF 10	27.2 ± 6.8[b]	15.5 ± 4.6[b]
RIF 10	14.4 ± 6.4	19.1 ± 13.9
SQV 10	3.9 ± 0.1	2.2 ± 1.0
SQV 10 + RIF 10	32.9 ± 4.9[a,b]	19.4 ± 8.3[b]

a, significantly different from RIF treated cells, $p \leq 0.05$.
b, significantly different from (corresponding) HIV-protease inhibitor treated cells, $p \leq 0.05$.

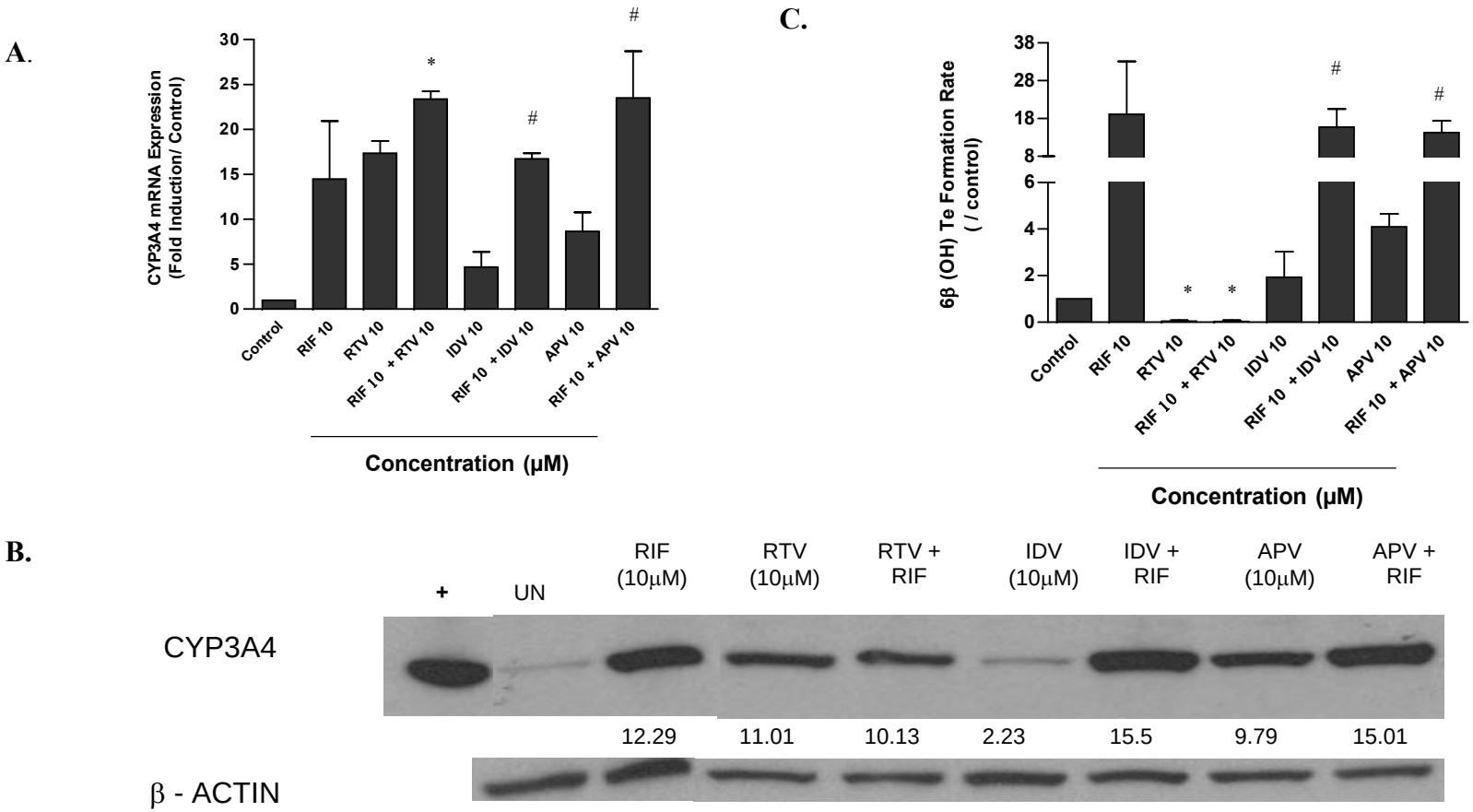


Figure 9. Interaction of protease inhibitors with rifampicin – effect on CYP3A4 mRNA, protein and activity

Hepatocytes were treated with RTV, IDV or APV alone (10 μM) or in combination with RIF (10 μM). CYP3A4 A) mRNA expression, B) protein content and C) activity were determined. The figure shows the mean of values obtained from three livers, with the S.D. indicated by the vertical bars. All mRNA and protein values are normalized to β-actin expression. *, significantly different from RIF treated cells; #, significantly different from protease inhibitor treated cells, $p \leq 0.05$.

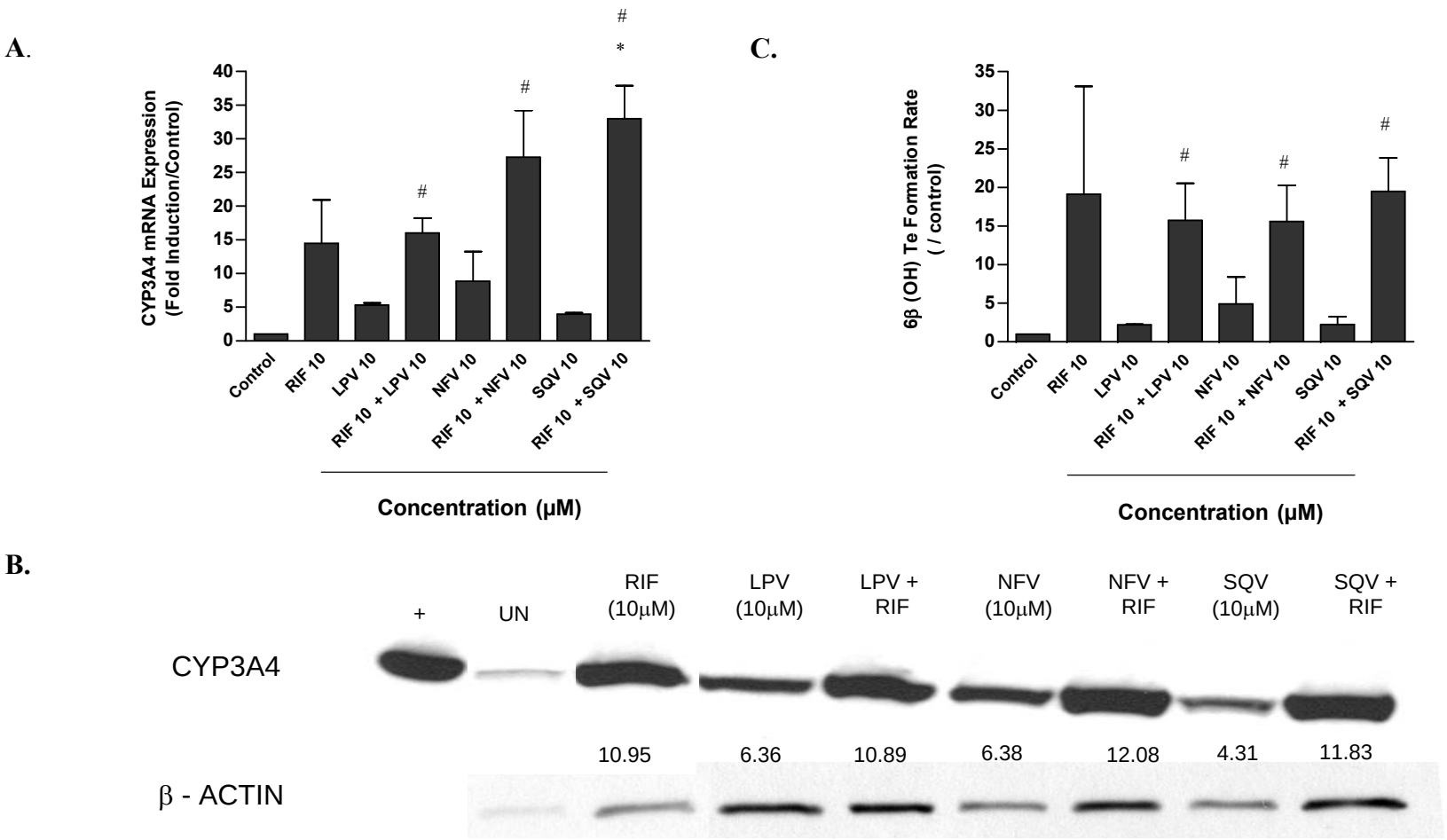


Figure 10. Interaction of protease inhibitors with rifampicin – effect on CYP3A4 mRNA, protein and activity

Hepatocytes were treated with LPV, NFV or SQV alone (10 μM) or in combination with RIF (10 μM). CYP3A4 A) mRNA expression, B) protein content and C) activity were determined. The figure shows the mean of values obtained from three livers, with the S.D. indicated by the vertical bars. All mRNA and protein values are normalized to β-actin expression. *, significantly different from RIF treated cells; #, significantly different from protease inhibitor treated cells, $p \leq 0.05$.

3.5.4. Effect of chronic treatment and washout of ritonavir and amprenavir on CYP3A4 expression and activity

To study the effect of chronic treatment and subsequent washout of drug from the hepatocyte system on CYP3A4 expression and activity, hepatocytes were first exposed to RTV or APV (1 and 10 μM) for 72 hours. At the end of this treatment period, the medium was replaced with HMM every 24 hours, for the next 72 hours. CYP3A4 mRNA expression, protein content and 6β(OH) TE formation rate were determined at the end of the treatment period and also at 24 hour intervals during the washout period.

The effect of exposure of hepatocytes to RTV (1 μM and 10 μM) for 72 hour followed by a 72 hours washout period, on CYP3A4 mRNA expression and activity, is summarized in Table 12 and Figure 11. Ritonavir treatment for 72 hourss resulted in increased CYP3A4 mRNA expression. This increase was 3.5 ± 0.4 and 9.6 ± 1.3 fold compared to control, at RTV 1μM and 10 μM, respectively. During the subsequent washout period, CYP3A4 mRNA expression decreased consistently until expression, after 72 hours of washout, was comparable to the control value.

Exposure of hepatocytes to 1 μM of RTV inhibited the CYP3A4 mediated TE metabolism to 0.291 ± 0.01-fold of control, while exposure to 10 μM RTV completely inhibited the CYP3A4 activity. During the washout period, the activity in the 1μM RTV treated cells recovered completely and became significantly higher than the control value after 72 hours of washout. In the 10 μM RTV treated cells, the activity gradually increased but remained significantly lower than the control value even after 72 hours.

The effect of exposure of hepatocytes to APV (1μM and 10 μM) for 72 hours followed by a 72 hours washout period, on CYP3A4 mRNA expression and activity, is summarized in

Table 13 and Figure 12. After 72 hours treatment, CYP3A4 expression was increased 4.9 ± 1.04 and 17.8 ± 1.7 fold compared to control, at APV 1 μM and 10 μM, respectively. Similarly, formation rate of 6β(OH) TE was increased 3.2 ± 1.1 and 11.3 ± 1.2 fold over control after 1 μM and 10 μM APV treatment for 72 hours. During the subsequent washout period, CYP3A4 mRNA expression as well as activity decreased consistently and were comparable to the control values after a 72-hour washout period. The APV effect after washout was similar to that seen with the prototypical inducer RIF (10 μM).

3.5.5. Calculation of $t_{1/2}$ and recovery time of CYP3A4 mRNA expression and activity

The CYP3A4 mRNA expression and activity values after 72 hours treatment with RTV and APV (1 and 10 μM), followed by a washout period were analyzed to calculate the $t_{1/2}$ and recovery time. Recovery time was defined as the total time elapsed post treatment to attain baseline mRNA expression and activity.

The recovery of CYP3A4 mRNA expression after RTV treatment followed first order kinetics, while the recovery of CYP3A4 activity followed zero order kinetics (Figure 11). The calculated $t_{1/2}$ value and recovery time for mRNA expression was 17.4 hours and 96.5 hours respectively (Table 14). Post RTV (10 μM) treatment, the CYP3A4 activity did not recover after a 72 hours washout and was predicted to recover completely after 244.7 hours. The decrease in mRNA expression and activity after APV (10 μM) treatment also followed first order kinetics (Figure 12). The $t_{1/2}$ and recovery time values are shown in Table 14.

Table 12. Effect of chronic ritonavir treatment followed by washout on CYP3A4 mRNA expression and activity

Treatment	Time (hours)	CYP3A4 mRNA expression (Fold / control)		6β (OH) Te formation rate (Fold / control)	
		RTV (1μM)	RTV (10μM)	RTV (1μM)	RTV (10μM)
+ RTV	72	3.59 ± 0.4^{c}	9.65 ± 1.3^{c}	0.2 ± 0.01^{c}	0.02 ± 0.0^{c}
RTV Wash out	24	4.03 ± 0.2^{c}	$19.0 \pm 3.4^{a,c}$	0.4 ± 0.09^{c}	$0.18 \pm 0.01^{a,c}$
	48	$2.64 \pm 0.1^{a,b,c}$	$6.1 \pm 1.1^{b,c}$	$1.3 \pm 0.1^{a,b,c}$	$0.32 \pm 0.04^{a,b,c}$
	72	$1.22 \pm 0.0^{a,b}$	$2.8 \pm 0.6^{a,b}$	$2.1 \pm 0.1^{a,b,c}$	$0.39 \pm 0.01^{a,b,c}$

Table 13. Effect of chronic amprenavir treatment followed by washout on CYP3A4 mRNA expression and activity

Treatment	Time (hours)	CYP3A4 mRNA expression (Fold / control)		6β (OH) Te formation rate (Fold / control)	
		APV (1μM)	APV (10μM)	APV (1μM)	APV (10μM)
+ APV	72	4.9 ± 1.0^{c}	17.8 ± 1.7^{c}	3.2 ± 1.1^{c}	11.3 ± 1.2^{c}
APV Wash out	24	$3.4 \pm 0.2^{a,c}$	$11.7 \pm 1.3^{a,c}$	2.0 ± 0.9	$5.2 \pm 0.2^{a,c}$
	48	$2.3 \pm 0.1^{b,c}$	$6.9 \pm 1.2^{a,b,c}$	1.3 ± 0.2^{a}	$2.5 \pm 0.05^{a,b,c}$
	72	$1.1 \pm 0.06^{a,b}$	$2.6 \pm 0.5^{a,b}$	1.5 ± 0.01	1.0 ± 0.002^{a}

a, significantly different from 72 hour treatment value, $p \leq 0.05$.
b, significantly different from 24 hour washout value, $p \leq 0.05$.
c, significantly different from control treatment for each day, $p \leq 0.05$. The baseline or control value was taken to be 1, since all the data was normalized to the expression or activity obtained with untreated cells, for each day.

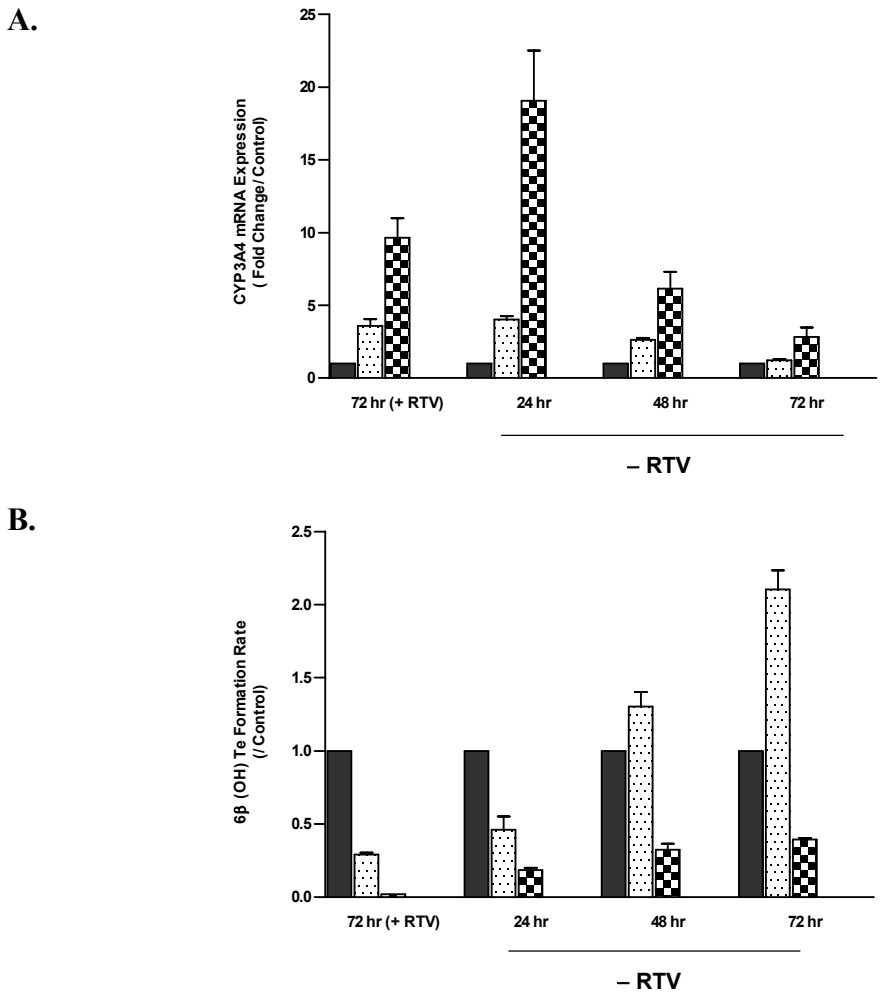


Figure 11. Effect of chronic ritonavir treatment followed by washout period on CYP3A4 mRNA expression, protein content and activity

Hepatocytes were treated with MeOH (closed bars), RTV 1 μM (dotted bars) and RTV 10 μM (checkered bars) for 72 hours. Medium was then changed with HMM every 24 hours for next 72 hours. CYP3A4 A) mRNA expression, B) protein content and C) activity were determined. The figure shows the mean of triplicate treatments, with the S.D. indicated by the vertical bars. All mRNA and protein values are normalized to β-actin expression. *, significantly different from control treatment, $p \leq 0.05$.

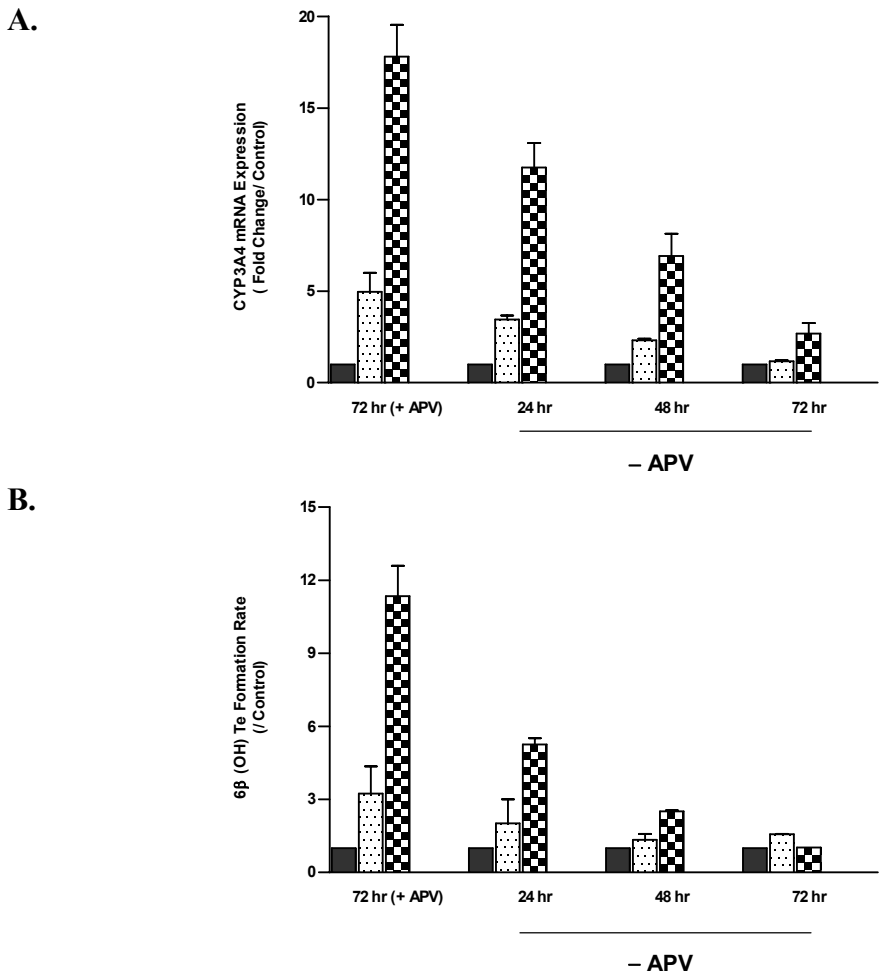


Figure 12. Effect of chronic amprenavir treatment followed by washout period on CYP3A4 mRNA expression, protein content and activity

Hepatocytes were treated with MeOH (closed bars), APV 1 μM (dotted bars) and APV 10 μM (checkered bars) for 72 hours. Medium was then changed with HMM every 24 hours for next 72 hours. CYP3A4 A) mRNA expression, B) protein content and C) activity were determined. The figure shows the mean of triplicate treatments, with the S.D. indicated by the vertical bars. All mRNA and protein values are normalized to β-actin expression. *, significantly different from control treatment, $p \leq 0.05$.

Table 14. Calculation of $t_{1/2}$ and recovery time of CYP3A4 mRNA expression and activity after treatment with ritonavir and amprenavir

Treatment	CYP3A4 mRNA expression (Fold / control)		6β (OH) Te formation rate (Fold / control)	
	$t_{1/2}$ (hours)	Recovery Time (hours)[a] (time to attain baseline expression)	$t_{1/2}$ (hours)	Recovery Time (hours)[a] (time to attain baseline activity)
RTV (10 μM)	17.4	96.5	-	244.7
APV (10 μM)	26.9	115.7	20.9	74

a, recovery time is equal to total time elapsed post 72 hour treatment to achieve baseline value.

3.6. Discussion

Most of the studies to date documenting the effect of HIV protease inhibitors on CYP3A4 have been carried out in microsomes or expressed enzyme systems. These systems are suited for studying inhibitory effects, but do not lend themselves well to evaluate the inductive potential of drugs. Primary cultures of human hepatocytes offer several advantages over these *in vitro* systems. Hepatocytes in culture offer an intact system containing all the necessary cofactors for oxidative, reductive and conjugative metabolism of drugs along with the various regulatory elements need to maintain and induce/inhibit the expression of enzymes. The effect of a variety of HIV protease inhibitors on CYP3A4 enzyme expression and activity has been extensively studied in this work.

In the first part of the current study, we evaluated the effect of increasing concentrations of three HIV protease inhibitors namely ritonavir, indinavir and amprenavir, on CYP3A4 mRNA

expression, protein content and activity measured as formation rate of 6β(OH) TE. The concentrations of protease inhibitors used in this study ranged from 0-10 μM, which are clinically relevant concentrations typically observed after oral administration of these drugs in humans (product Monographs).

Human hepatocytes when exposed to ritonavir (0–10 μM), showed a concentration dependent increase in CYP3A4 mRNA expression (EC_{50} = 0.9 μM) comparable to that of prototypical CYP3A4 inducer RIF (EC_{50} = 0.5 μM) (Sahi et al., 2000). The maximum magnitude (E_{max}) of CYP3A4 mRNA induction with RTV was also comparable to that of RIF. The increase in mRNA expression resulted in a moderate (2.5-4 folds) increase in CYP3A4 protein content. Luo et. al. have reported a similar increase in CYP3A4 mRNA expression (20-fold) and protein content (1.5-fold) when human hepatocytes were exposed to 10μM RTV (Luo et al., 2002).

A significant observation in our study was the complete inhibition of the CYP3A4 activity despite a large increase in CYP3A4 mRNA expression and a moderate increase in protein content. In the present study, ritonavir inhibited the CYP3A4 mediated metabolism of testosterone (IC_{50} = 0.0197 μM). This IC_{50} value is in agreement with the previously reported inhibitory constants for RTV in human liver microsomes (IC_{50} = 0.017 μM) (Kumar et al., 1996).

Complete inhibition of CYP3A4 enzyme activity by RTV and appearance of somewhat smeared CYP3A4 protein bands after RTV treatment, suggests the possibility of covalent binding of RTV to CYP3A4 thus making the enzyme 'nonfunctional'. This possibility of mechanism-based inhibition or inactivation of CYP3A4 in presence of RTV is supported by the study of (Kumar et al., 1996), that documents the high affinity of RTV for CYP3A4 binding sites.

The present study showed no correlation between mRNA levels of CYP3A4 and PXR after RTV treatment. Thus, the increase in mRNA expression post RTV treatment is not attributable to PXR mRNA expression. An earlier study conducted by (Luo et al., 2002) has shown a correlation between CYP3A4 mRNA expression and PXR activation, after RTV treatment (10 μM) in human hepatocytes. However, to the best of our knowledge, there are no reports in the literature that document increased PXR mRNA expression after RTV treatment, although PXR activation is seen in a reporter gene assay system.

Treatment with IDV did not result in significant changes in mRNA expression of CYP3A4, while CYP3A4 mediated testosterone metabolism was inhibited (IC_{50} = 0.33 μM). This shows that IDV is a less potent inhibitor of CYP3A4 compared to RTV, which is in agreement with the studies done in human liver microsomes.

In all previous studies, APV was reported to be a weak inhibitor of CYP3A4 mediated metabolism. In our study, chronic treatment of human hepatocytes with APV (0-10 μM) showed increased mRNA expression, protein content and activity of CYP3A4. At a 10 μM concentration, the increase in mRNA expression with APV is 31% of that observed with RIF (10 μM). Similarly, the increase in activity with APV (10 μM) is 20% of that observed with RIF (10 μM).

Based upon the present study, it can be concluded that in human hepatocytes, ritonavir and indinavir inhibit CYP3A4 activity, ritonavir being more potent inhibitor than indinavir. On the other hand, amprenavir acts as CYP3A4 inducer. In summary, it is shown that HIV protease inhibitors have differential effects on CYP3A4 expression and activity. Consistent with the observations in human hepatocytes, RTV administration decreased metabolism of immunosuppressive drugs. One example is the interaction between tacrolimus and Kaletra, a

combination of ritonavir and lopinavir. After initiation of Kaletra in HIV-positive patient after transplantation, tacrolimus half-life was significantly increased (20 days) thus requiring discontinuation of tacrolimus for 3-5 weeks, in order to maintain the required tacrolimus blood concentration levels. This interaction was found to be more significant compared to that observed with single protease inhibitor nelfinavir, where the tacrolimus half-life was increased to 10.3 days (Jain et al., 2003).

Patients taking HIV protease inhibitors take multiple medications most of which are metabolized by CYP3A4. Thus the modulation of CYP3A4 expression and activity by protease inhibitors can possibly lead to drug interactions. Rifampicin was chosen to study the drug-drug interaction with protease inhibitors, since it is a potent PXR activator and a known inducer of CYP3A4. Rifampicin induces CYP3A4 by increasing mRNA, protein and CYP3A4 activity. We showed that RTV, by itself, also increases CYP3A4 expression and hence might be expected to enhance the effect of rifampicin, when given in combination. On the contrary, we observed a predominant inhibition of CYP3A4 activity by RTV, thereby completely masking the induction effect of RIF. The inactivation of CYP3A4 enzyme activity in the presence of RTV may be responsible for loss of RIF mediated induction of CYP3A4. In the presence of RTV, the inductive effect of RIF was completely masked. This observation predicts the dominance of its inhibitory effect on CYP3A4 activity even in presence of potent inducers.

For the other protease inhibitors, namely amprenavir, indinavir, lopinavir and saquinavir when given in combination with RIF, it was clearly observed that the effect on mRNA expression, protein and CYP3A4 activity is dominated by RIF. These protease inhibitors are known to be less potent than RTV in inhibiting CYP3A4 activity and hence do not modulate the induction effect of RIF.

Amprenavir, by itself, showed a significant increase in CYP3A4 activity and hence was expected to accentuate the inductive effect of RIF, when given in combination. It was observed in this study that the combination effect of APV and RIF was not additive, which could potentially be due to the saturable (capacity limited) nature of CYP3A4 enzyme.

This study predicts that the inhibitory effect of protease inhibitor on CYP3A4 predominates during chronic treatment. However, after sudden withdrawal, there is no more inhibition but the CYP3A4 system may remain induced for a few days. Based upon this prediction, we treated human hepatocytes with RTV and APV for three days and studied the withdrawal effect of protease inhibitors on CYP3A4 expression and activity by washing the drugs out for a further 72 hours. This study showed that CYP3A4 mRNA expression increased during treatment with RTV (1 and 10 μM) and exhibited first order decline during the washout period with $t_{1/2}$ of 17.4 hours. During the treatment period, RTV completely inhibited the CYP3A4 activity at 1 and 10 μM. During the washout period, the CYP3A4 activity in cells treated with 1μM RTV, recovered to the baseline value following zero order kinetics in 48 hours with a further increase in activity post 72 hours of washout. In 10 μM RTV treated cells, the recovery process followed zero order kinetics but was delayed and a total of 244 hours washout period is predicted for complete recovery to the baseline value. It can be speculated that physical binding of RTV at the CYP3A4 enzyme site results in metabolically inactive enzyme. Removal of RTV from the enzyme-binding site along with synthesis of new mRNA and protein, results in recovery of CYP3A4 metabolic activity back to the control value.

A similar washout study with APV showed increased mRNA expression and activity during the treatment period. The decline in expression and activity, after removal of APV, followed first order kinetics with $t_{1/2}$ = 26.9 hours and 20.9 hours for mRNA expression and

activity, respectively. The prototypical inducer RIF showed similar decrease in CYP3A4 expression and activity after chronic treatment followed by a washout period.

In summary, this is the first study to the best of our knowledge, showing a concentration dependence effect of individual protease inhibitors on CYP3A4 expression and activity in a human hepatocyte system. Secondly, the interaction potential of these protease inhibitors with currently known CYP3A4 substrates and inducers was studied. Thirdly, the reversible nature of CYP3A4 inhibition or induction by protease inhibitors after treatment and removal has been shown. The calculation of kinetic parameters such as $t_{1/2}$ and recovery time for CYP3A4 expression and activity after drug discontinuation can help further understanding of the time course of the effect of protease inhibitors on CYP3A4.

This systematic study characterizing the effect of protease inhibitors on CYP3A4 enzymes will also help in better understanding of the clinically observed complex drug-drug interactions with other CYP3A4 substrate drugs. With reference to immunosuppressive drugs, the effect of RTV will be seen immediately as an inhibition of metabolism and will persist as long as RTV is in use. When RTV is stopped, the recovery of activity of CYP3A4 involved in the metabolism will be restored to normal values over a four day time period. Careful adjustment in the dose of immunosuppressive rugs is warranted during this time period. To the best of our knowledge, the greatest inhibition of immunosuppressive drug metabolism is seen with ritonavir and this does contributes to a significant reduction in dose of immunosuppressive drugs in transplant patients.

4. Effect of HIV protease inhibitors on hepatic phase II drug metabolizing enzyme in human hepatocytes

4.1. Abbreviations

APV	Amprenavir
CAR	Constitutive androgen receptor
CYP	Cytochrome P450
E3G	Estradiol-3-glucuronide
HMM	Hepatocyte maintenance medium
IDV	Indinavir
LPV	Lopinavir
NFV	Nelfinavir
PB	Phenobarbital
PCHH	Primary cultures of human hepatocytes
PXR	Pregnane X receptor
RIF	Rifampicin
RTV	Ritonavir
SQV	Saquinavir
UGT	UDP-glucuronosyl transferase

4.2. Abstract

Aims: Amongst hepatic phase II drug metabolizing enzymes, UGT1A1 is an important isoform, responsible for the glucuronidation of endogenous compounds such as bilirubin, estradiol and testosterone and of drugs like mycophenolic acid, acetaminophen commonly used in transplant patients. The use of the HIV-protease inhibitor indinavir is associated with unconjugated hyperbilirubinemia thought to be due to inhibition of UGT1A1 by indinavir. UGT1A1 is known to be transcriptionally regulated by nuclear hormone receptors PXR and CAR. We hypothesized that HIV- protease inhibitors will modulate the expression of UGT1A1 by altering their regulatory pathways. In the present study we have evaluated the effect of different HIV protease inhibitors on UGT1A1 expression and activity using primary cultures of human hepatocytes.

Methods: To determine the effect on UGT1A1 Expression and activity, PCHH were exposed to ritonavir (RTV), indinavir (IDV) and amprenavir (APV) (0-10 μM) for 72 hours. Quantitative Real-time PCR technique was used to determine UGT1A1 mRNA expression, while UGT1A1 protein was determined using western blotting. Estradiol was used as a probe substrate to determine UGT1A1 activity.

Results: Ritonavir (1-10 μM) significantly increases the mRNA expression of UGT1A1 (EC_{50}= 0.857 μM), while increasing the protein content up-to 2 fold. UGT1A1 activity was increased after RTV treatment (EC_{50}= 0.473 μM). Indinavir increases UGT1A1 mRNA expression 2-fold, but no significant changes in protein content were observed. Indinavir at lower concentrations (≤ 0.5μM) increased UGT1A1 activity, while it inhibited UGT1A1 activity by 43% at the highest concentration (10 μM). Amprenavir treatment resulted in increased mRNA expression, a 2-fold increase in protein content and a 1.6-fold increase in UGT1A1 activity. Though the basal mRNA expression of UGT1A1 was not variable among the livers used, a great deal of variability in the inducibility of UGT1A1 was observed after treatment with the prototypical inducer PB and also after treatment with ritonavir and amprenavir.

Conclusions: This work shows that HIV-protease inhibitors have differential effects on the expression and activity of UGT1A1. The modulation of UGT1A1 activity by HIV protease inhibitors could possibly affect the clearance of drugs and endogenous compounds metabolized by glucuronidation.

4.3. Introduction

Hepatic phase II metabolizing enzymes, such as UDP-glucoronosyltransferases (UGTs), play a pivotal role in glucuronidation of several drugs namely mycophenolic acid, acetaminophen and morphine, all of which are used in transplant patients. Factors modulating the expression and activity of UGTs will impact clearance of these drugs and will contribute to the observed variability in the pharmacokinetics of immunosuppressive drugs.

UGTs are also responsible for glucuronidation of several endogenous compounds such as bilirubin, estradiol and testosterone. Bilirubin, the major product of heme catabolism, is cleared from the circulation by conjugation to glucuronic acid in hepatocytes by the microsomal enzyme UDP-glucuronosyltransferase (UGT) and then by secretion into bile. In HIV infected patients, it has been reported that the use of protease inhibitors such as indinavir and atazanavir, is associated with 6-25% incidence of asymptomatic, unconjugated hyperbilirubinemia in the absence of histological liver injury, which often leads to development of clinical jaundice and interruption of HIV treatment. Of the total 15 human UGT isoforms identified so far, UGT1A1 is considered to be exclusively responsible for bilirubin glucuronidation Thus, it is believed that elevated unconjugated bilirubin concentration after indinavir treatment is due to inhibition of UGT1A1 enzyme.

Unconjugated hyperbilirubinemia in rats after administration of indinavir was attributed to direct competitive inhibition of UGT1A1 by indinavir. Experiments carried out in expressed enzyme system and human liver microsomes indicate a direct inhibition of UGT1A1 mediated bilirubin glucuronidation by atazanavir and indinavir (Zhang et al., 2005). A strong association between UGT1A1*28 allele and risk of developing unconjugated hyperbilirubinemia while intaking indinavir, has also been reported (Zucker et al., 2001; Rotger et al., 2005). Since

previous studies reporting the inhibition of UGT1A1 after treatment with HIV protease inhibitors have been conducted in rat or human microsomes, the potential for the induction of UGT1A1 enzyme has not been evaluated.

The phase II metabolizing enzyme UGT1A1 is transcriptionally regulated by the nuclear orphan receptor such as pregnane X receptor (PXR) and constitutive androstane receptor (CAR). *In vitro* studies have shown that ritonavir is a potent activator of PXR, though PXR activating potential of other HIV protease inhibitors is not yet reported.

We hypothesized that HIV protease inhibitors will 'indirectly' modulate the expression and activity of UGT1A1 enzyme, by affecting the expression of regulatory pathways such as the expression of PXR and CAR. The aim of this study was to use primary cultures of human hepatocytes to characterize the effect of HIV protease inhibitors namely ritonavir, indinavir and amprenavir on UGT1A1 expression and activity. UGT1A1 was selected as a prototype UGT enzyme, as it is the primary enzyme required for the glucuronidation of most drugs.

4.4. Methods

4.4.1. Hepatocyte treatment protocol to study the effect of HIV protease inhibitors on expression and activity of UGT1A1

To determine the effect of HIV protease inhibitors on UGT1A1 mediated metabolism and on the expression of various hepatic drug transporters, twenty-four hours after plating, cells were exposed to RTV, IDV and APV (0 - 10 μM) for 72 hours. On the day of the study, cells were washed with 1.5 ml of fresh medium for 1 hour. To measure UGT1A1 enzyme activity, cells were then incubated in medium containing 200μM estradiol for an additional 60 minutes. At the end of that time, medium along with cell lysate was sampled and stored at -80°C for estradiol-3-

glucuronide (E-3-G) determination by HPLC (Alkharfy and Frye, 2002). Cells were also harvested in phosphate buffer (0.1 M, pH 7.4) and stored at -80°C for protein determination and detection of immunoreactive UGT1A1 protein (Lowry O, 1951). The relative amounts of proteins were measured by the intensity of immunoblot staining carried out by densitometry (ImageJ, v1.34, http://reb.info.nih.gov/ij)

Cells were also harvested for mRNA by adding 1 mL of Trizol reagent to each well of a 6-well plate. The RNA samples were stored at -20°C for Real Time PCR analysis. The PCR procedure used is described in Chapter 2 (Materials and methods) Primers used for UGT1A1, PXR, CAR and β-actin detection are described in Table 8. The relative cDNA content in each sample was determined from standard curves constructed from serially diluted human cDNA samples. The mRNA expression for each gene was normalized to β-actin in each sample and expressed as fold change over control treatment.

4.4.2. Data analysis

The data were analyzed using a one-way analysis of variance with a post hoc Tukey's multiple comparison procedure. A p value of ≤ 0.05 was considered statistically significant. All statistical analysis was performed using PRISM software version 4.0 (GraphPad Software, Inc., San Diego, CA, USA).

Table 15. Donor information for human hepatocyte preparations used in Chapter 4

Donor HH #	Age	Sex[a]	Race[b]	Cause of death[c]	Drug History	Viability	Percoll separation
1117	68y	F	C	ICH/ Stroke	Labetolol, Verapamil, Clonidine	82	No
1118	73y	F	C	Head trauma	Atenolol, Imipramine	80	No
1184	66y	F	C	-	None reported	87	Yes
1210	35y	F	C	Head trauma	Dopamine, Vasopressin, hydrocortisone	83	Yes
1218	50y	F	C	CA/ Anoxia	-	85	No

[a]F, female; [b]C, Caucasian; [c] ICH, intra cranial hemorrhage; CA, cardiac arrest

4.5. Results

Hepatocytes from a total of 5 liver donors were used to conduct the experiments outlined in this Chapter. Their relevant demographics, drug history and cell viability information is cited in Table 15.

Chronic exposure of PCHH to RTV, IDV and APV (0- 50 μM) resulted in cellular toxicity at concentrations greater than or equal to 20 μM (Figure 4, Chapter 3). Therefore, to study the effect on UGT1A1 expression and activity, protease inhibitors at concentrations ≤ 10 μM were used. To determine the effect of increasing concentrations of RTV, IDV and APV on UGT1A1 enzyme expression and activity, cells were exposed to RTV, IDV or APV (0, 0.1, 0.5, 1, 5 and 10 μM) for 72 hours. PB (2mM), a prototypical inducer of UGT1A1 enzyme, was used as a positive control in all the experiments.

4.5.1. Effects of ritonavir on UGT1A1 expression and activity

Ritonavir treatment resulted in a significant increase in UGT1A1 mRNA expression, in a concentration dependent manner (EC_{50} = 0.857 ± 0.038 μM) (Figure 13A). The increase in UGT1A1 mRNA expression varied considerably among different hepatocyte isolates, with 3.9 to 14.2-fold increase over control being seen after RTV (10 μM) treatment. Ritonavir (0.1 and 0.5 μM) did not alter UGT1A1 protein content but concentrations of 1, 5 and 10 μM increased UGT1A1 protein content up to 2-fold (Figure 13B). The formation rate of E-3-G significantly increased in a concentration dependent manner after treatment with RTV (EC_{50}= 0.473 μM), indicating increased UGT1A1 activity (Figure 13C). The increase in E-3-G formation rate observed was 2.7 ± 0.07 and 2.2 ± 0.09 fold, at 5 and 10 μM RTV, respectively.

4.5.2. Effects of indinavir on UGT1A1 expression and activity

Treatment with IDV (0-10 μM) increased the UGT1A1 mRNA expression 2-fold compared to control and this effect was not concentration dependent (Figure 14A). Indinavir did not alter the UGT1A1 protein content significantly at the concentrations used in this study (Figure 14B). Since there was not much induction in UGT1A1 mRNA expression, less inter-individual variability was observed after IDV treatment. Indinavir at lower concentrations (0.1 and 0.5μM) increased E-3-G formation rate 1.66 and 1.37-fold over control, while at higher concentration (10μM) the formation rate of E-3-G was reduced to 57.2 ± 1.59 % of control (Figure 14C).

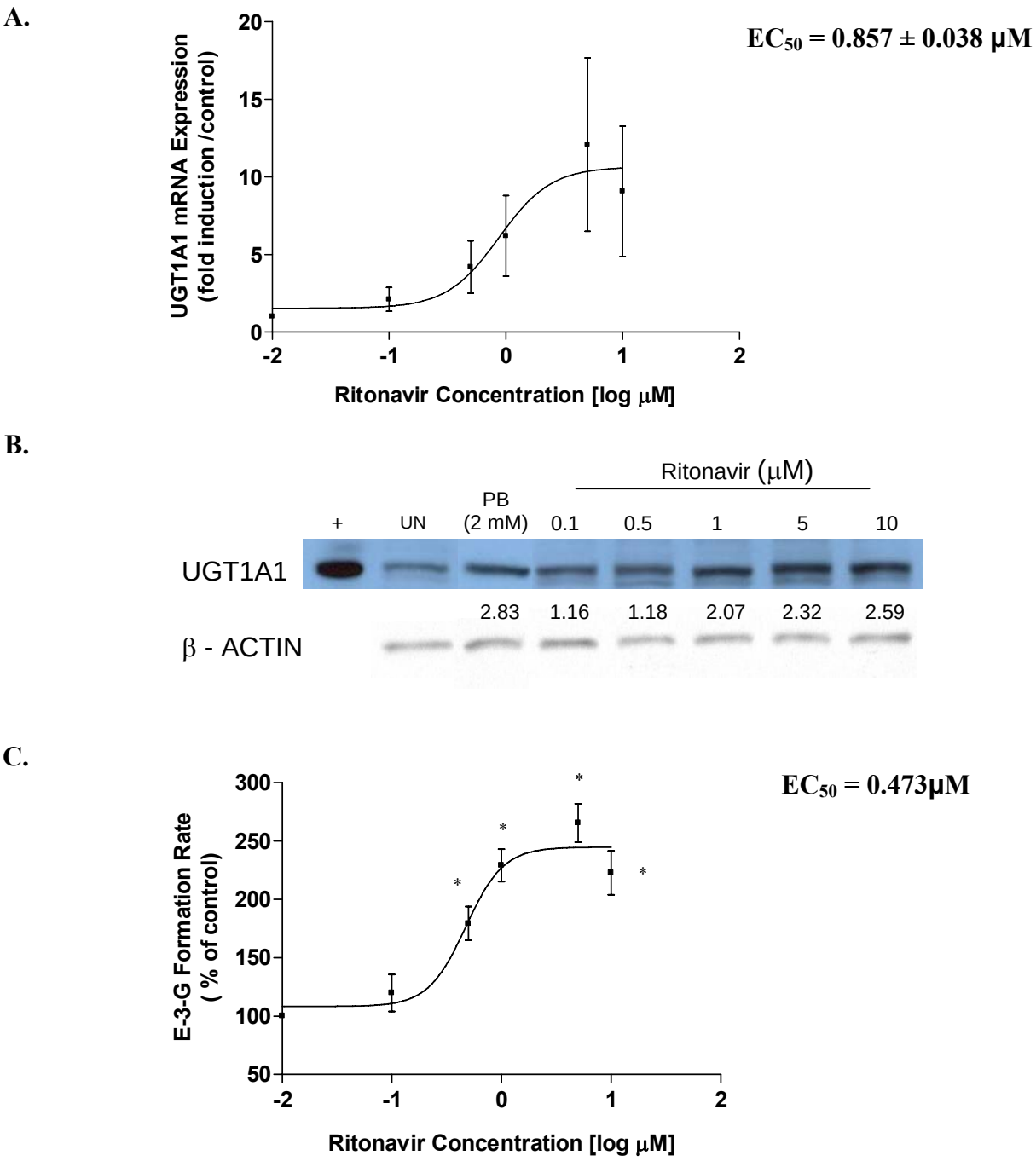
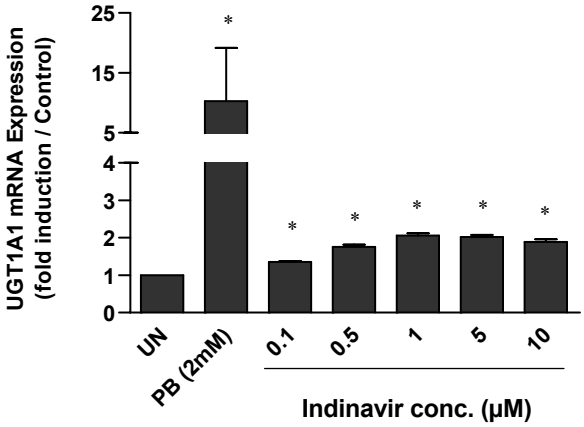


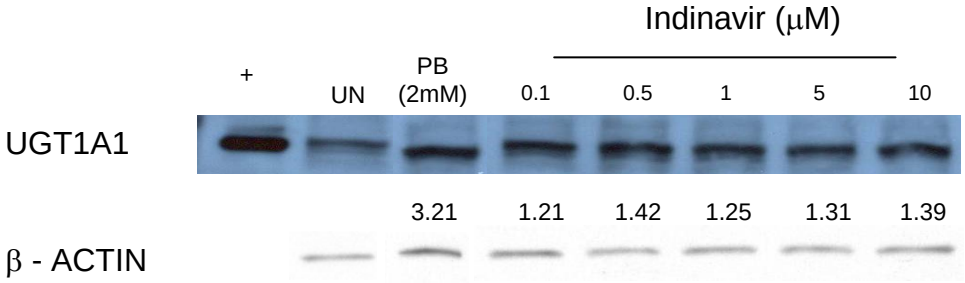
Figure 13. Effect of ritonavir on UGT1A mRNA expression, protein content and activity

Hepatocytes were exposed to RTV (0 - 10 µM) for 72 hours and UGT1A1 A) mRNA expression, B) protein content and C) activity were determined. Each figure shows the mean of values obtained from three livers, with the S.D. indicated by the vertical bars. All mRNA and protein values are normalized to β-actin expression. *, significantly different from vehicle treated cells, $p \leq 0.05$

A.



B.



C.

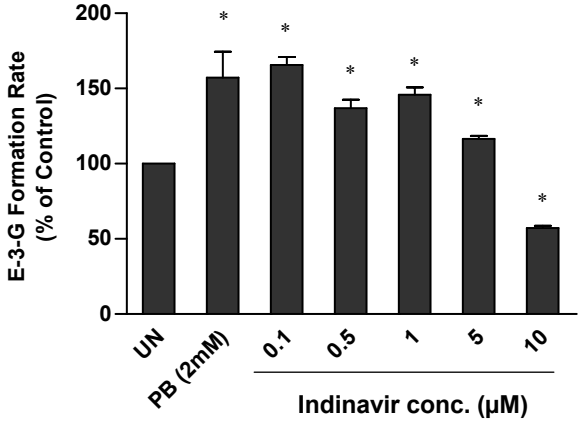


Figure 14. Effect of indinavir on UGT1A mRNA expression, protein content and activity

Hepatocytes were exposed to IDV (0 - 10 μM) for 72 hours and UGT1A1 A) mRNA expression, B) protein content and C) activity were determined. Each figure shows the mean of values obtained from three livers, with the S.D. indicated by the vertical bars. All mRNA and protein values are normalized to β-actin expression. *, significantly different from vehicle treated cells, $p \leq 0.05$

4.5.3. Effects of amprenavir on UGT1A1 expression and activity

Hepatocytes treated with APV (0-10 μM) showed a significant increase in UGT1A1 mRNA expression, in concentration dependent manner. Similar to the effect seen after RTV treatment, the increase in UGT1A1 mRNA expression was considerably variable within the different hepatocyte isolates tested; with 3.1 to 16.8-fold induction seen after treatment with highest concentration of APV (10 μM) (Figure 15A). A 2-fold increase in the UGT1A1 protein content was seen at ATV concentrations 5 and 10 μM (Figure 15B). The formation rate of E-3-G was not altered at lower concentrations of APV (0-5 μM), where as the 10 μM APV increased the E-3-G formation rate 1.6 ± 0.07 fold over control (Figure 15C)

The basal mRNA expression of UGT1A1 was not variable among the hepatocytes obtained from different donors (CT = 20.78 ± 0.89). But, a large inter-individual variability was seen in the inducibility of UGT1A1 mRNA expression. Cells when exposed to PB (2 mM) showed 4.0 to 16.6-fold increase in UGT1A1 mRNA expression compared to vehicle treated cells (Figure 15). PB (2 mM) treatment increased UGT1A1 protein content 2-fold; while estradiol-3-glucuronide (E-3-G) formation rate was increased 1.97 ± 0.17- fold over control treatment.

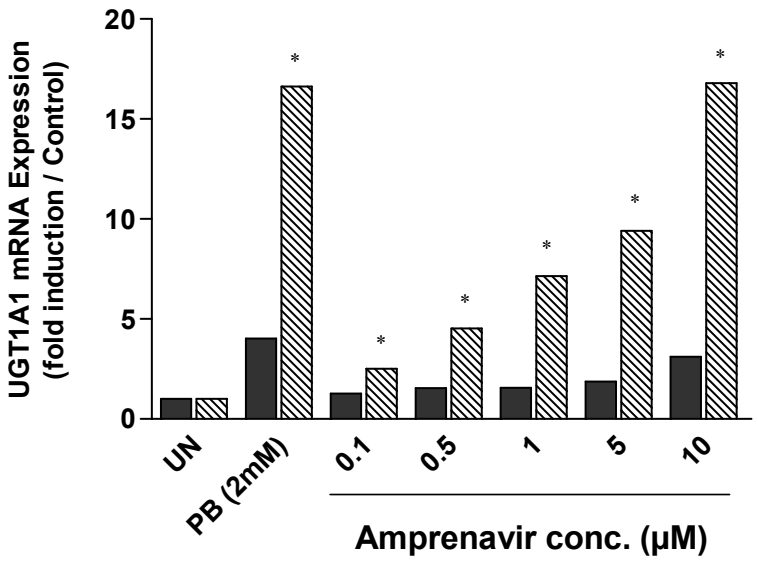
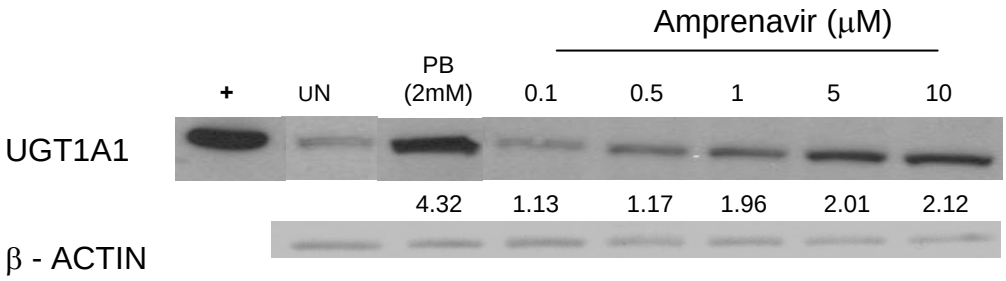


Figure 15. Effect on UGT1A1 mRNA induction after chronic treatment with amprenavir

Hepatocytes were treated with PB (2mM) or APV (0-10 μM) for 72 hours and UGT1A1 mRNA expression was determined. The figure represents data from two donors, HH1184 showing least UGT1A1 mRNA induction (black bars) and HH1210 showing maximum UGT1A1 mRNA induction (diagonal thatched bars). All mRNA values are normalized to β-actin expression in each sample. *, Significantly different from untreated control cells for each liver, $p \leq 0.05$.

A.



B.

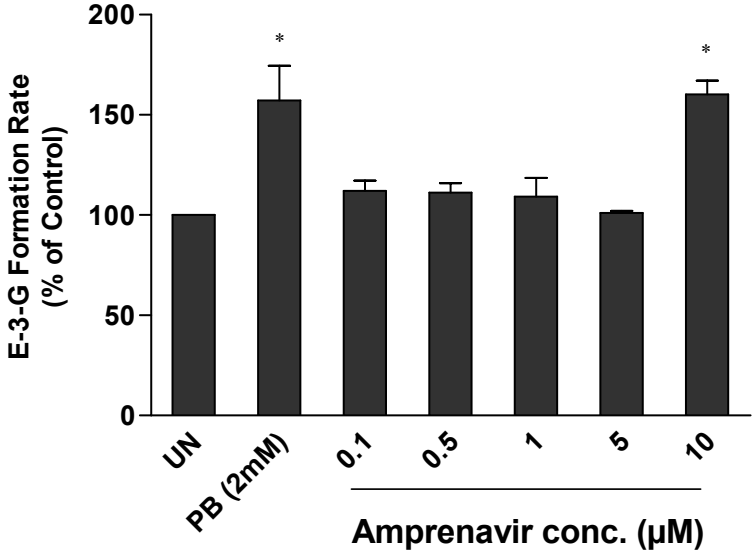


Figure 16. Effect of amprenavir treatment on UGT1A1 protein content and activity

Hepatocytes were treated with APV (0-10 μM) for 72 hours and UGT1A1 A) protein content and B) activity were determined. The figure shows a representative immunoblot from HH1210. All protein values are normalized to β-actin expression. The estradiol-3-glucuronide formation rate values are the mean of treatments from three livers, with the S.D. indicated by the vertical bars. *, significantly different from vehicle treated cells, $p \leq 0.05$

4.5.4. Correlation between mRNA expression of UGT1A1 and nuclear receptors after treatment with protease inhibitors

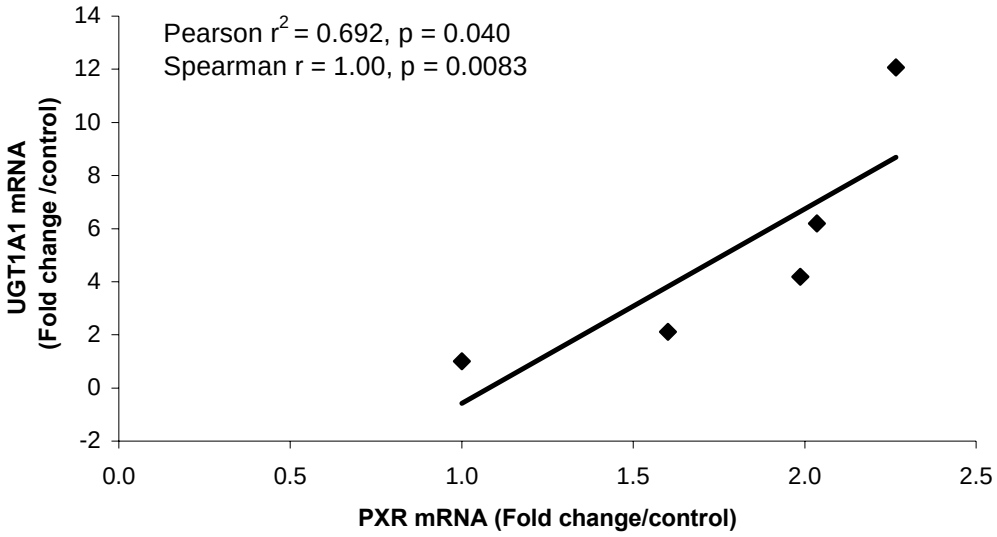
To determine if the induction of UGT1A1 mRNA expression after treatment with RTV, IDV and APV is due to increased mRNA expression of nuclear receptors such as PXR and CAR, a correlation analysis was carried out using PRISM software version 4.0 (GraphPad Software, Inc., San Diego, CA, USA).

Figure 17A shows that after RTV (0-10 μM) treatment, the increase in UGT1A1 mRNA expression correlated well with a corresponding increase in PXR mRNA expression (Pearson's correlation coefficient, r^2=0.692, p=0.040 or Spearman's correlation coefficient, ρ =1.00, p =0.0083) while there was a lack of correlation between UGT1A1 and CAR mRNA expression (Pearson's correlation coefficient, r^2=0.057, p=0.349 or Spearman's correlation coefficient, ρ =0.3, p =0.3417) (Figure 17B).

After treatment with IDV (Figure 18A), there was a lack of correlation between UGT1A1 and PXR mRNA expression (Pearson's correlation coefficient, r^2=0.56, p=0.067 or Spearman's correlation coefficient, ρ =0.71, p =0.068). Additionally, no correlation was observed between UGT1A1 and CAR mRNA expression (Pearson's correlation coefficient, r^2=0.579, p=0.068 or Spearman's correlation coefficient, ρ =0.7, p =0.116) after treatment with IDV (0-10 μM) as shown in Figure 18B.

Increased UGT1A1 mRNA expression, after APV treatment (0-10 μM), correlated well with increased PXR mRNA expression (Pearson's correlation coefficient, r^2=0.651, p=0.026 or Spearman's correlation coefficient, ρ =0.943, p =0.0083) and also with increased CAR expression (Pearson's correlation coefficient, r^2=0.899, p=0.002 or Spearman's correlation coefficient, ρ =0.943, p =0.0083) (Figure 19A and Figure 19B, respectively).

A.



B.

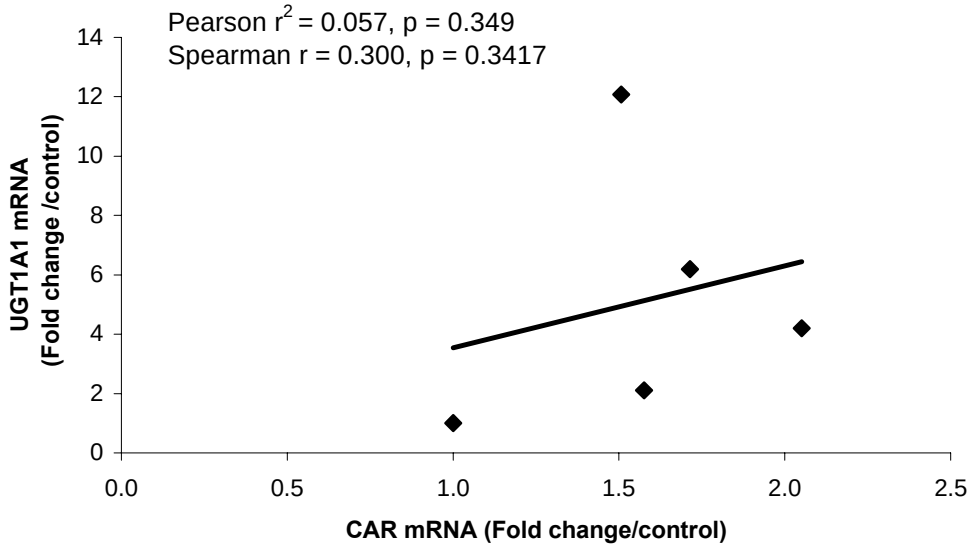
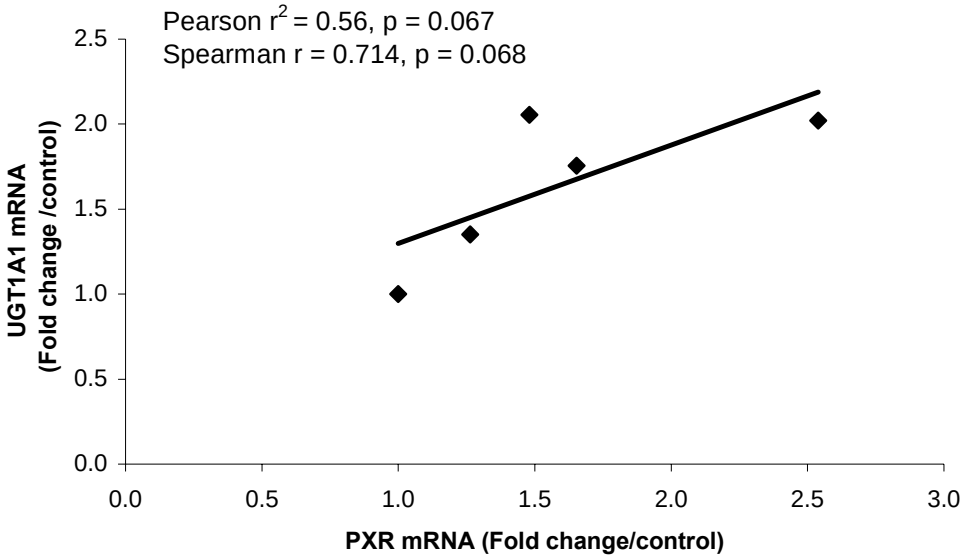


Figure 17. Correlation between the induction of expression of nuclear receptors and UGT1A1 mRNA after ritonavir treatment

Hepatocytes were treated with RTV (0-10 μM) for 72 hours and mRNA expression of UGT1A1, PXR and CAR was determined. The figure represents the correlation between UGT1A1 mRNA induction and A) PXR mRNA induction and B) CAR mRNA induction. Each value represents the mean of treatments from three livers. All mRNA values are normalized to β-actin expression. Correlations were measured by Pearson's correlation coefficient (r) and Spearman's Rho (ρ). A p value of 0.05 was considered statistically significant.

A.



B.

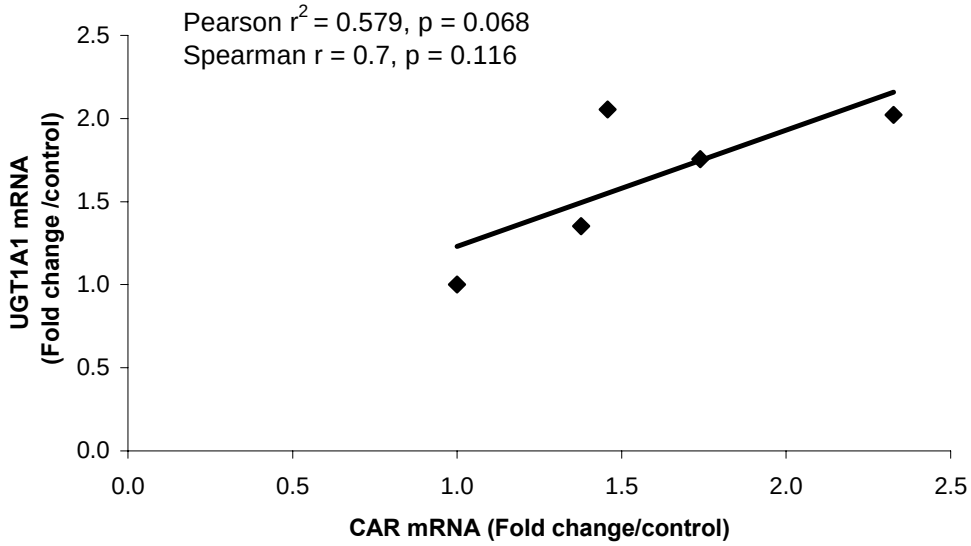
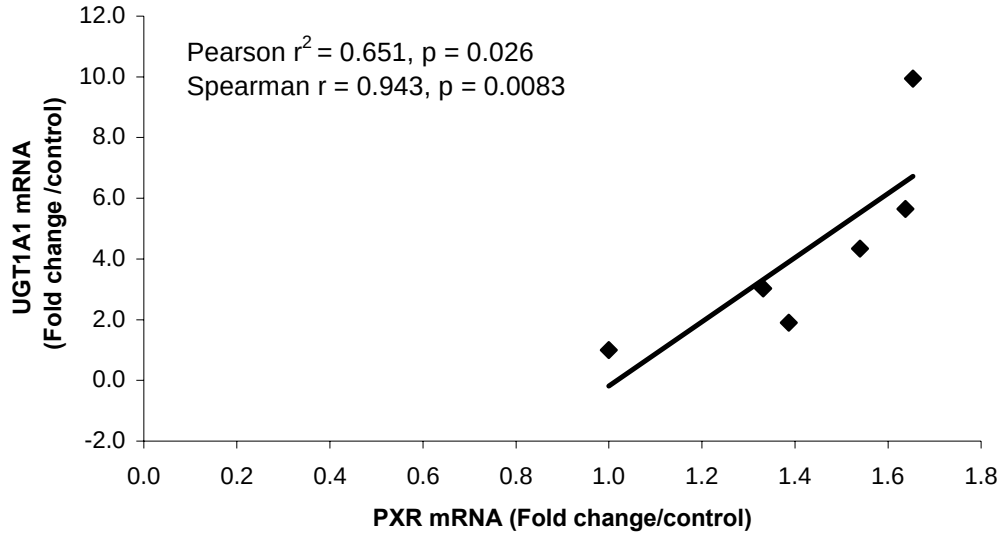


Figure 18. Correlation between the induction of expression of nuclear receptors and UGT1A1 mRNA after indinavir treatment

Hepatocytes were treated with IDV (0-10 μM) for 72 hours and mRNA expression of UGT1A1, PXR and CAR was determined. The figure represents the correlation between UGT1A1 mRNA induction and A) PXR mRNA induction and B) CAR mRNA induction. Each value represents the mean of treatments from three livers. All mRNA values are normalized to β-actin expression. Correlations were measured by Pearson's correlation coefficient (r) and Spearman's Rho (ρ). A p value of 0.05 was considered statistically significant.

A.



B.

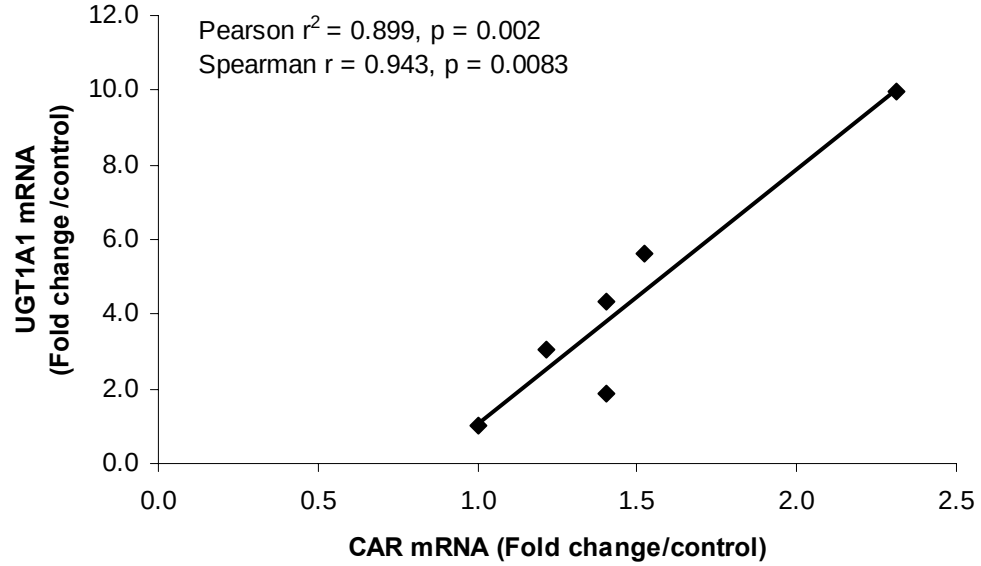


Figure 19. Correlation between the induction of expression of nuclear receptors and UGT1A1 mRNA after amprenavir treatment

Hepatocytes were treated with APV (0-10 μM) for 72 hours and mRNA expression of UGT1A1, PXR and CAR was determined. The figure represents the correlation between UGT1A1 mRNA induction and A) PXR mRNA induction and B) CAR mRNA induction. Each value represents the mean of treatments from three livers. All mRNA values are normalized to β-actin expression. Correlations were measured by Pearson's correlation coefficient (r) and Spearman's Rho (ρ). A p value of 0.05 was considered statistically significant.

4.6. Discussion

Primary cultures of human hepatocytes represent an intact and physiologically relevant model useful for studying the modulation of hepatic UGT1A1 in humans. Studies carried out in expressed enzymes or microsomes cannot be used to evaluate the regulation of UGTs.

Currently there are very few studies demonstrating the effect of HIV protease inhibitors on human UGT1A1 enzyme expression and activity. The present study carried out using primary cultures of human hepatocytes addresses various issues related to the effect of protease inhibitors on UGT1A1 enzyme, including concentration dependent effects of protease inhibitors, interindividual variability and correlations with the expression of nuclear receptors such as PXR and CAR.

UGT1A1 catalytic activities were determined by the quantification of estradiol-3-glucuronide (E-3-G) formation rate. Estradiol was chosen as a specific probe substrate for hepatic UGT1A1 catalytic activity based on previous work demonstrating that E-3-G was a selective UGT1A1 metabolite in human liver (Soars et al., 2004).

In the present study, we have shown that ritonavir increases the UGT1A1 mRNA expression in a concentration dependent manner (EC_{50} = 0.857 ± 0.038 μM), with a maximum 3.93 to 14.21-fold increase over control after RTV (10 μM) treatment. Recently, Smith et al. have also observed large inducing effects (> 17-fold) on UGT1A1 mRNA levels after treatment with 10 μM RTV in human hepatocyte culture system. We observed about a 2-fold increase in the UGT1A1 protein content after RTV treatment which was consistent with the previous report showing a more than 2-fold increase in UGT1A1 protein at 10 μM RTV concentration (Smith et al., 2005). We observed a concentration dependent increase in UGT1A1 catalytic activity as reflected by the increased formation rate of estradiol-3-glucuronide (EC_{50}= 0.473 μM). The EC_{50}

values for UGT1A1 activity after treatment with a prototypical inducers namely RIF, 3-MC and PB, have been reported to be 0.4 μM, 0.8 μM and 107 μM, respectively (Smith et al., 2005). After comparing the EC_{50} values, RTV can be considered as a potent inducer of hepatic UGT1A1 expression and activity. The observed increase in UGT1A1 activity in PCHH after RTV treatment was contradictory to the report by Zhang et.al. They have reported inhibition of human UGT1A1 enzyme expressed in baculovirus-infected insect cells by RTV (IC_{50} = 19 μM) (Zhang et al., 2005). This discrepancy may be due to the lack of regulatory cofactors and nuclear receptors in the expressed enzyme model system used.

There are no previous reports demonstrating the effect of amprenavir on UGT1A1 mediated metabolism in human hepatocytes. In our study, amprenavir increased the mRNA expression 3.1 to 16.8-fold, while a 2-fold increase in the UGT1A1 protein content was observed. At the highest concentration used in the present study, APV (10 μM) increased the E-3-G formation rate 1.6-fold, comparable to that seen after PB (2 mM) and RTV (10 μM).

Indinavir, on the other hand, showed only a slight increase in UGT1A1 mRNA expression (2-fold), with no significant changes in UGT1A1 protein expression. Surprisingly, IDV at lower concentrations increased E-3-G formation rate (1.66-fold) comparable to that of PB (2 mM), while significantly inhibiting the UGT1A1 mediated estradiol metabolism to 57.2 % at 10 μM concentration. Since UGT1A1 mRNA and protein expression was not altered, this inhibition at a higher concentration of IDV is considered to be a 'direct' effect on the UGT1A1 enzyme. Zhang et al. have reported a similar inhibitory effect of IDV on UGT1A1 activity (IC_{50} = 87 μM) (Zhang et al., 2005). There are no literature reports documenting that HIV-protease inhibitors are good substrates of UGT enzymes, barring one study where a glucuronide metabolite of indinavir has been identified. Indinavir is suggested to bind UGT1A1 and act as partial competitive

inhibitor of UGT1A1 (Balani et al., 1996). Thus, the direct inhibition of UGT1A1 activity at higher concentrations of IDV observed in our study using human hepatocytes has precedence. This observation also supports the occurrence of hyperbilirubinemia after indinavir treatment to be mediated by inhibition of UGT1A1 enzyme.

We observed that the basal mRNA expression of UGT1A1 was consistent among the various hepatocyte cultures used (CT = 20.78 ± 0.89), but the increase in UGT1A1 mRNA expression after treatment with the prototypical inducer PB (2mM) or after treatment with RTV and APV varied considerably (3 to 18-fold). Other investigators have reported large inter-individual differences in hepatic UGT1A1 expression and/or activity. Smith et al. have reported 35-fold and 6-fold differences in UGT1A1 protein levels and catalytic activity, respectively (Smith et al., 2005). Though no information was available regarding the UGT1A1 genotype of the liver donors, the possibility of polymorphism in the promoter region of UGT1A1 gene cannot be excluded and hence warrants further study.

It has been recently discovered that UGT1A1 contains both PXR and CAR binding motifs in its promoter region (Sugatani et al., 2001; Xie et al., 2003). Thus, similar to CYP3A4 enzyme, UGT1A1 is also believed to be under transcriptional control. Ritonavir is shown to be a potent PXR activator (Luo et al., 2002), while there are no reports documenting indinavir or amprenavir as PXR activators. We observed a good correlation between increased UGT1A1 mRNA and increased PXR mRNA expression after RTV treatment, while there was no correlation with changes in CAR mRNA expression. Increased UGT1A1 mRNA expression after amprenavir treatment showed a good correlation with increased PXR as well as increased CAR expression. We thus predict amprenavir to be an activator of PXR as well as CAR, though further studies measuring PXR activation after amprenavir treatment will be needed to support this observation.

A poor correlation was seen in UGT1A1 expression and PXR or CAR expression in presence of IDV. This observation supports our previous finding that IDV did not have any effect on UGT1A1 mRNA expression but inhibits UGT1A1 activity by direct binding to the enzyme.

In summary, our studies document the effect of ritonavir, indinavir and amprenavir on the expression and activity of UGT1A1 enzyme. This is the first study to the best of our knowledge to report the effect of increasing concentrations of protease inhibitors on UGT1A1 mediated metabolism in human hepatocytes, and to document EC_{50} values for UGT1A1 induction after RTV treatment. Furthermore, our studies have demonstrated the use of primary cultures of human hepatocytes in clarifying induction or inhibition discrepancies due to absence of cofactors in other *in vitro* model systems. We also demonstrated that the effect of ritonavir and amprenavir on UGT1A1 is 'indirect' and is possibly mediated through nuclear receptors, while the inhibitory effect of indinavir on UGT1A1 activity does not appear to involve regulation by PXR and CAR. This study also shows that protease inhibitors can significantly modulate the expression and activity of UGT1A1 thereby affecting the clearance of drugs and endogenous compounds metabolized by glucuronidation.

UGT1A1 being the primary enzyme involved in the glucuronidation of drugs used in transplant patients such as acetaminophen, morphine and mycophenolic acid, modulation of UGT1A1 expression and activity by HIV-protease inhibitors will alter glucuronidation of these drugs. Our observations indicate that HIV-protease inhibitors have a moderate effect on UGT1A1 expression and activity and other factors should also be considered while explaining the large variability in pharmacokinetics of the drugs used in liver transplant patients.

5. Effect of HIV protease inhibitors on the expression of hepatic drug transporters in human hepatocytes

5.1. Abbreviations

APV	amprenavir
BSEP	bile salt export pump
CAR	constitutive androgen receptor
HPIs	HIV protease inhibitors
HMM	hepatocyte maintenance medium
IDV	indinavir
MDR1	multidrug resistant protein 1
MRP2	multidrug resistance associated protein 2
MRP6	multidrug resistance associated protein 6
PCHH	primary cultures of human hepatocytes
PXR	pregnane X receptor
P-gp	P-glycoprotein
RIF	rifampicin
RTV	ritonavir

5.2. Abstract

Aims: Hepatic canalicular membrane transporters such as P-glycoprotein (P-gp), multidrug resistance protein 2 (MRP2) and bile salt export pump (BSEP) are involved in removal of drugs and endogenous substances from the blood circulation. Immunosuppressive agents are substrates for these transporters. Thus alterations in expression and activity of transporters are thought to alter the pharmacokinetics of immunosuppressive agents. The objective of the present study was to use primary cultures of human hepatocytes (PCHH) to study the concentration-dependent effect of HIV-protease inhibitors, namely ritonavir, indinavir and amprenavir, on the expression of canalicular transporters, namely P-gp, MRP2, MRP6 and BSEP. Additionally, the possible involvement of pregneane X receptor (PXR) and constitutive androgen receptor (CAR) in the regulation of drug transporters after treatment with HIV-protease inhibitors was also studied.

Methods: To determine the effect of concentration, hepatocytes coated with Matrigel^{TM} (0.233 mg/ml) were exposed to ritonavir (RTV), indinavir (IDV) and amprenavir (APV) (0-10 μM) for 72 hours. The mRNA expression of various hepatic transporters such as MDR1, MRP2, MRP6 and BSEP and that of nuclear receptors, PXR and CAR was measured by quantitative Real-time PCR. The mRNA expression of each gene was normalized to β-actin in each sample and expressed as fold change over control treatment.

Results: After ritonavir treatment, mRNA expression of MDR1, MRP2 and BSEP was increased and correlated well with increased PXR expression, while no correlation with CAR expression was observed. Similarly, after APV treatment, increase in mRNA expression of MDR1 and MRP2 was correlated with PXR but not with CAR mRNA expression. Decrease in mRNA expression of MRP6 after RTV treatment was associated with increased PXR mRNA expression. But, no changes in MRP6 mRNA expression were observed after indinavir and amprenavir treatment.

Conclusions: This study has demonstrated that increasing concentrations of HIV-protease inhibitors have differential effects on expression of drug efflux transporters namely P-gp (MDR1), MRP2, MRP6 and BSEP in a human hepatocyte system. The modulation of transporter expression is possibly mediated via mechanisms involving nuclear receptors such as PXR. Given the magnitude of changes in the transporter expression observed in this study due to HIV-

protease inhibitors treatment, it can be concluded that effects of HIV-protease inhibitors on immunosuppressive drugs is mainly due to alteration of CYP3A4, rather than transporters.

5.3. Introduction

Hepatic canalicular membrane transporter proteins such as P-glycoprotein (MDR1), multidrug resistance associated proteins (MRP2, MRP6) and bile salt export pump (BSEP) are efflux transporters involved in the removal of endogenous substances such as bile acids and exogenous substances such as drugs used in transplantation namely cyclosporine, tacrolimus and mycophenolic acid. Factors inhibiting or inducing these transporters can therefore affect the intracellular concentration of drugs, thereby altering pharmacokinetics and pharmacodynamic profiles of the drugs or of endogenous substances, affecting the normal physiological processes in the liver.

HIV protease inhibitors administered in conjunction with immunosuppressive agents show significant potential for drug-drug interactions, which can be mediated through alteration in drug transporters. A strong induction of P-glycoprotein expression by ritonavir, amprenavir and nelfinavir in the human colon carcinoma cell line has been documented (Perloff et al., 2003). A recent report has documented the inhibition of human breast cancer resistance protein (BCRP) by ritonavir, saquinavir and nelfinavir (Gupta et al., 2004). Additionally, ritonavir, saquinavir and indinavir have been shown to be high affinity substrates for P-glycoprotein and MRP2 (Kim et al., 1998; Huisman et al., 2002; Williams et al., 2002). Interestingly, along with drug metabolizing enzymes like CYP3A4, hepatic efflux transporters such as P-glycoprotein and MRP2 are also transcriptionally regulated by the nuclear orphan receptors pregnane X receptor (PXR) and constitutive androgen receptor (CAR). *In vitro* studies have shown that ritonavir is a potent activator of PXR (Luo et al., 2002). Since protease inhibitors modulate PXR activity and CYP3A4 enzyme, they might also alter expression and activity of several drug transporters.

There are no reports documenting the effect, if any, of these protease inhibitors on the expression of efflux transporters in primary cultures of human hepatocytes. Based on this background, we hypothesize that HIV protease inhibitors will alter the expression of hepatic drug transporters by modulating the expression of PXR and CAR involved in their regulation. Thus, the aim of this study was to use human hepatocytes in culture to characterize the effect of increasing concentrations of HIV protease inhibitors such as ritonavir, indinavir and amprenavir on the expression of canalicular drug transporters namely P-glycoprotein, MRP2, MRP6 and BSEP.

5.4. Methods

5.4.1. Hepatocyte treatment protocol to study the effect of HIV protease inhibitors on expression of hepatic drug transporters

Twenty-four hours after plating, hepatocytes were coated with Matrigel^{TM} (0.233 mg/ml). Cells were then exposed to RTV, IDV and APV (0 - 10 μM) for 72 hours in order to determine the effect on the expression of hepatic drug transporters namely P-glycoprotein, MRP2, MRP6 and BSEP and on nuclear receptors PXR and CAR.

Cells were harvested for mRNA by adding 1 mL of Trizol reagent to each well of a 6-well plate. The RNA samples were stored at -20°C for Real Time PCR analysis. The PCR procedure and primers used for MDR1, MRP2, MRP6, BSEP, PXR, CAR and β-actin detection are described in Chapter 2 (Materials and Methods). The relative cDNA content in each sample was determined from standard curves constructed from serially diluted human cDNA samples. The mRNA expression for each gene was normalized to β-actin in each sample and expressed as fold change over control treatment.

5.4.2. Data analysis

All values were calculated as mean ± S.D. The data were analyzed using a one-way analysis of variance with a post hoc Tukey's multiple comparison procedure. A p value of ≤ 0.05 was considered statistically significant. All statistical analysis as well as the correlation analysis was performed using PRISM software version 4.0 (GraphPad Software, Inc., San Diego, CA, USA).

Table 16. Donor information for human hepatocyte preparations used in Chapter 5

Donor HH #	Age	Sex[a]	Race[b]	Cause of death[c]	Drug History	Viability	Percoll separation
1112	69y	F	C	Anoxia/ CA	Dopamine	80	No
1117	68y	F	C	ICH/ Stroke	Labetolol, Verapamil, Clonidine	82	No
1118	73y	F	C	Head trauma	Atenolol, Imipramine	80	No
1184	66y	F	C	-	None reported	87	Yes
1210	35y	F	C	Head trauma	Dopamine, Vasopressin, hydrocortisone	83	Yes
1218	50y	F	C	CA/ Anoxia	-	85	No

[a]F, female; [b]C, caucasian; [c]CA, cardiac arrest; ICH, intra cranial hemorrhage

5.5. Results

Hepatocytes from a total of 6 liver donors were used to conduct the experiments outlined in this Chapter. Their relevant demographics, drug history and cell viability information is cited in Table 16.

Chronic exposure of hepatocyte culture to RTV, IDV and APV (0- 50 μM) resulted in cellular toxicity at concentrations greater than or equal to 20 μM (Figure 4, Chapter 3). Thus, in this study all protease inhibitors were used at concentrations ≤ 10 μM. To determine the effect of increasing concentrations of protease inhibitors on expression of transporters, cells were exposed to RTV, IDV or APV (0, 0.1, 0.5, 1, 5 and 10 μM) for 72 hours. RIF (10 μM) was used as a positive control in all the experiments.

5.5.1. Effect of ritonavir on mRNA expression of MDR1, MRP2, MRP6 and BSEP

Figure 20 shows the effect of RTV on the mRNA expression of MDR1, MRP2, MRP6 and BSEP. Ritonavir (10μM) showed 8.2 ± 0.3-fold and 2.6 ± 0.6-fold increase in the mRNA expression of MDR1 and MRP2, respectively. Additionally, the increase in mRNA expression of MDR1 and MRP2 was dependent on RTV concentration. BSEP mRNA expression was increased to 2-fold up to 5 μM RTV concentration, but was found not to be significantly different from the control value at 10μM. On the other hand, with increasing concentrations of RTV, MRP6 expression gradually decreased to 60.6 % of control value at 10μM concentration.

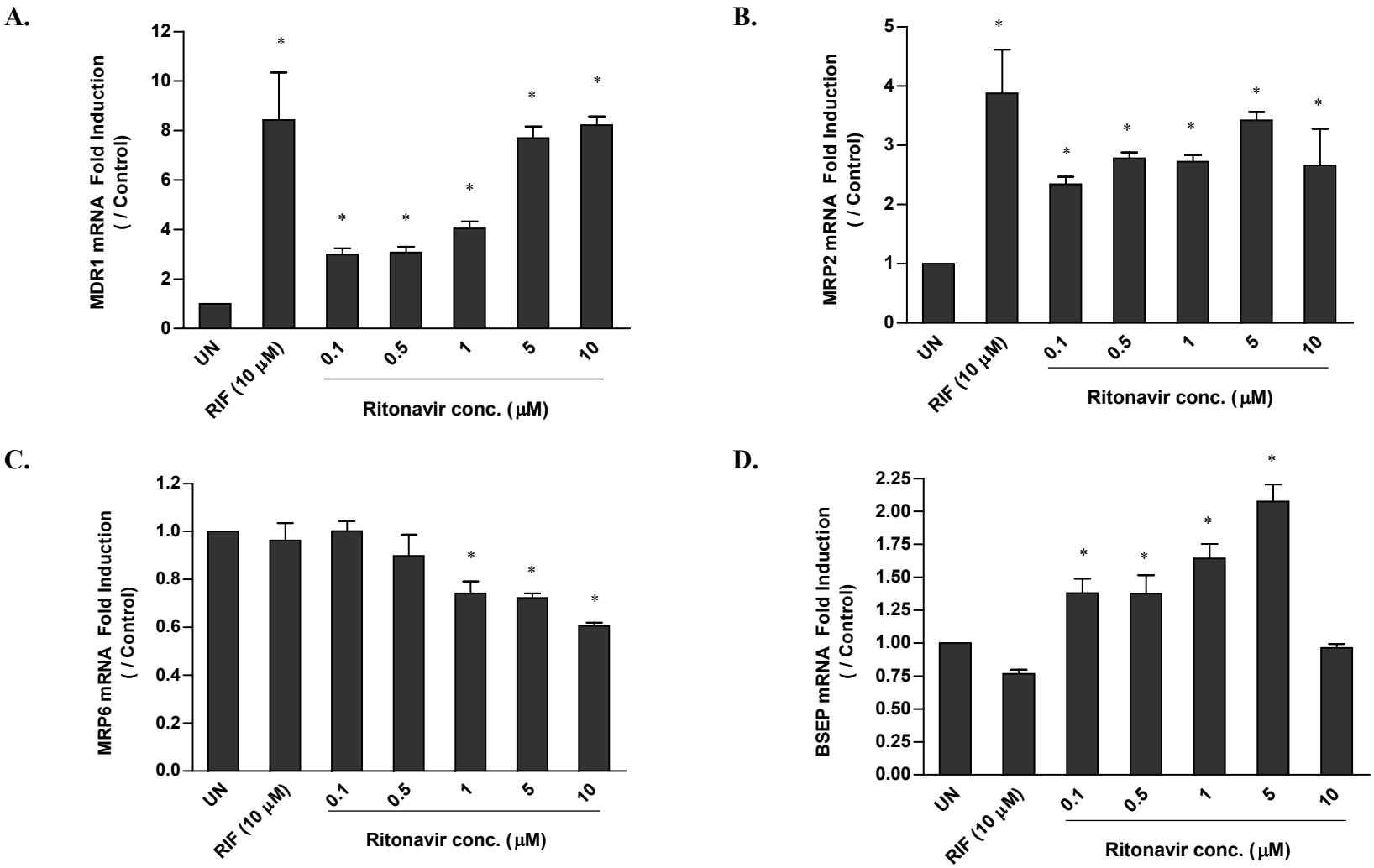


Figure 20. Effect of ritonavir on mRNA expression of hepatic drug transporters

Hepatocytes were treated with RTV (0 –10 μM) and mRNA expression of A) MDR1, B) MRP2, C) MRP6 and D) BSEP was measured. The figure shows the mean of values obtained from four donors, with the S.D indicated by vertical bars. All mRNA values are normalized to β-actin expression. *, significantly different from vehicle treated cells, $p \leq 0.05$.

5.5.2. Effect of indinavir on mRNA expression of MDR1, MRP2, MRP6 and BSEP

Indinavir increased the mRNA expression of MDR1, MRP2 and BSEP up to 2-fold, but this effect was not observed to be concentration dependent. Indinavir did not show any significant changes in the mRNA expression of MRP6 (Figure 21).

5.5.3. Effect of amprenavir on mRNA expression of MDR1, MRP2, MRP6 and BSEP

Figure 22 shows that after treatment with APV, a concentration dependent increase in MDR1 and MRP2 mRNA expression was observed. APV (10μM) showed 4.0 ± 0.2-fold and 1.9 ± 0.07-fold increase in the mRNA expression of MDR1 and MRP2, respectively. BSEP mRNA expression was significantly increased (1.3-fold over control) only at the highest concentration of APV (10μM). There was no significant effect on MRP6 mRNA expression after APV treatment.

The positive control RIF (10μM) used in this study increased MDR1 and MRP2 mRNA expression 8.4 ± 1.9-fold and 3.8 ± 0.7-fold of control value, respectively. MRP6 mRNA expression was not altered, while BSEP mRNA expression was decreased to 76.6 % of control after RIF (10 μM) treatment.

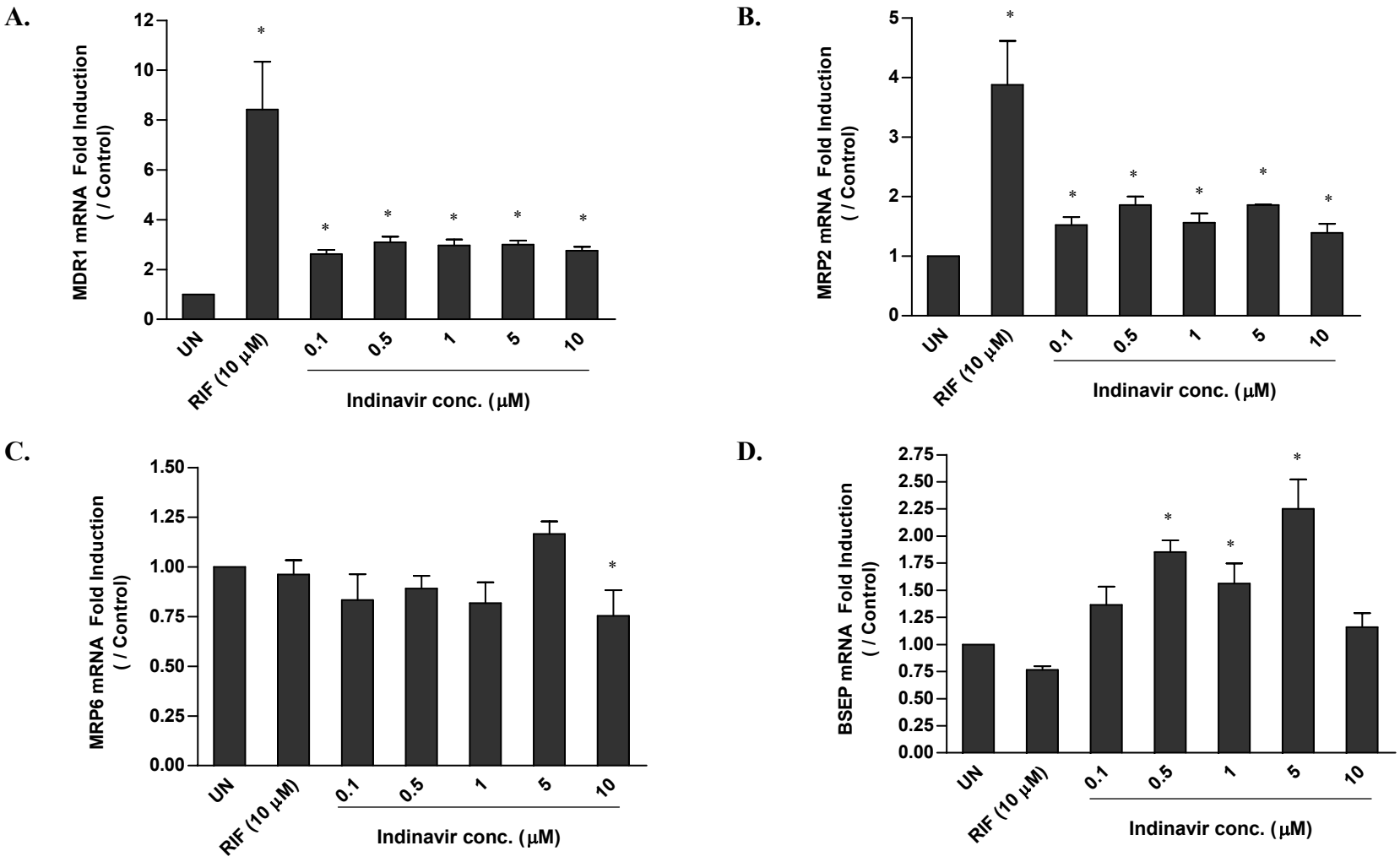


Figure 21. Effect of indinavir on mRNA expression of hepatic drug transporters

Hepatocytes were treated with IDV (0 –10 μM) and mRNA expression of A) MDR1, B) MRP2, C) MRP6 and D) BSEP was measured. The figure shows the mean of values obtained from four donors, with the S.D indicated by vertical bars. All mRNA values are normalized to β-actin expression. *, significantly different from vehicle treated cells, $p \leq 0.05$.

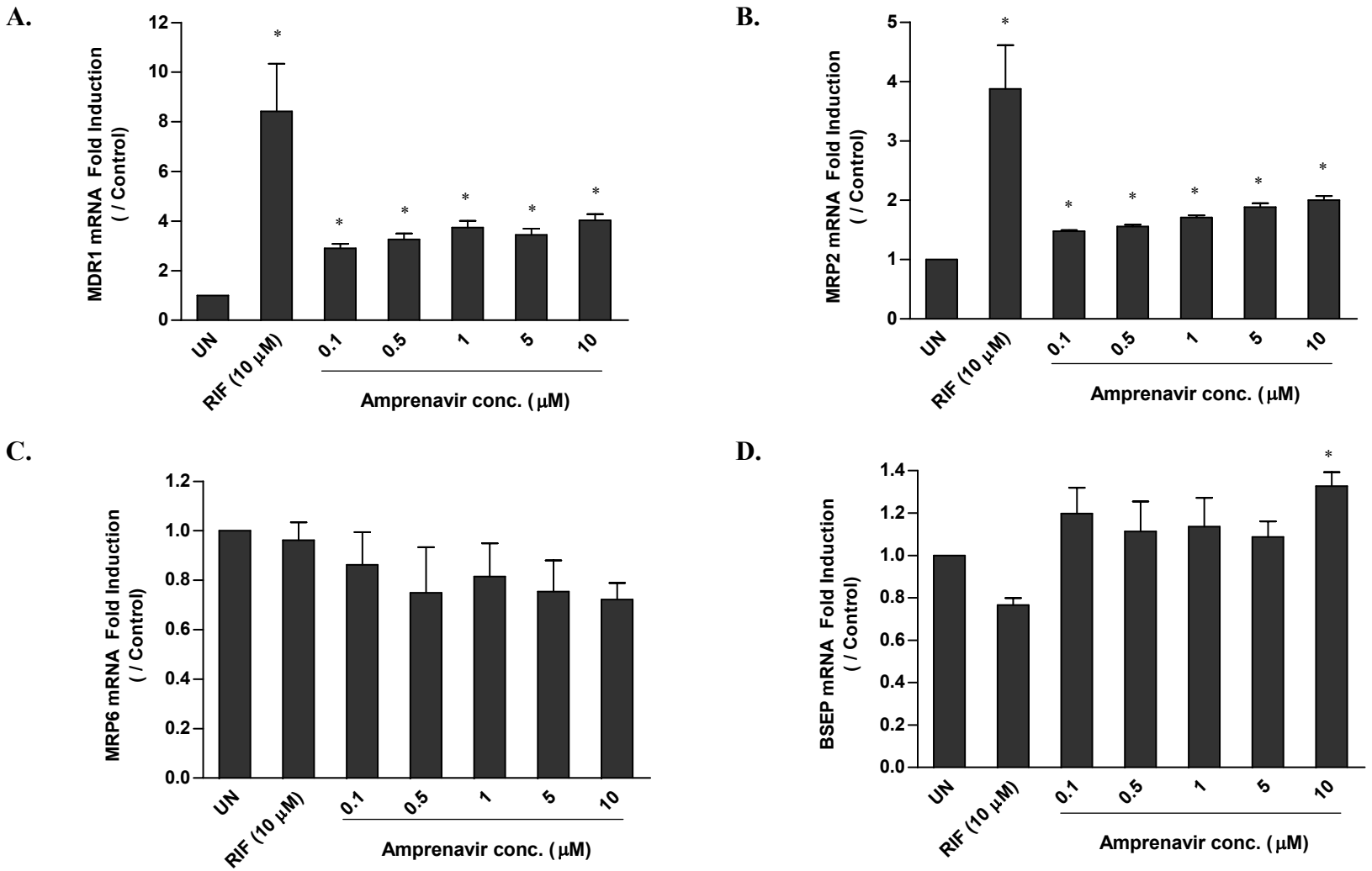


Figure 22. Effect of amprenavir on mRNA expression of hepatic drug transporters

Hepatocytes were treated with APV (0 –10 μM) and mRNA expression of A) MDR1, B) MRP2, C) MRP6 and D) BSEP was measured. The figure shows the mean of values obtained from four donors, with the S.D indicated by vertical bars. All mRNA values are normalized to β-actin expression. *, significantly different from vehicle treated cells, $p \leq 0.05$

5.5.4. Correlation between mRNA expression of transporters and nuclear receptors after treatment with protease inhibitors

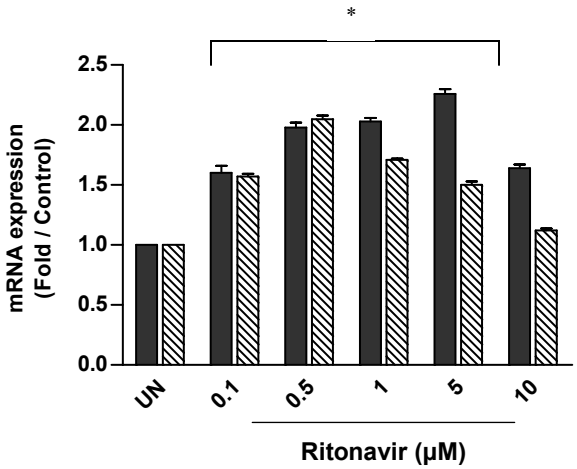
The mRNA expression of PXR and CAR was increased up to 2.5-fold after treatment with RTV, IDV and APV treatment (0-10μM) (Figure 23).

To determine if the changes in the mRNA expression of transporters after treatment with RTV, IDV and APV were due to changes in the mRNA expression of nuclear receptors, PXR and CAR, a correlation analysis was carried out using PRISM software version 4.0 (GraphPad Software, Inc., San Diego, CA, USA). Table 17 and Table 18 show the corresponding correlation coefficients for PXR and CAR, respectively. After RTV (0-10 μM) treatment, increase in mRNA expression of MDR1, MRP2, and BSEP was associated with a corresponding increase in PXR mRNA expression. On the other hand, changes in mRNA expression of MDR1, MRP2, MRP6 and BSEP did not correlate with changes in CAR expression after RTV treatment (Figure 24 and Figure 25).

After IDV (0-10 μM) treatment, mRNA expression of MRP2 and BSEP correlated well with increased PXR and CAR mRNA expression, while MDR1 and MRP6 expression was not correlated with either PXR or CAR mRNA expression (Figure 26 and Figure 27, respectively)

After APV (0-10 μM) treatment, mRNA expression of MDR1 and MRP2 correlated well with PXR mRNA expression, while only MRP2 and BSEP expression correlated well with CAR expression (Figure 28 and Figure 29).

A.



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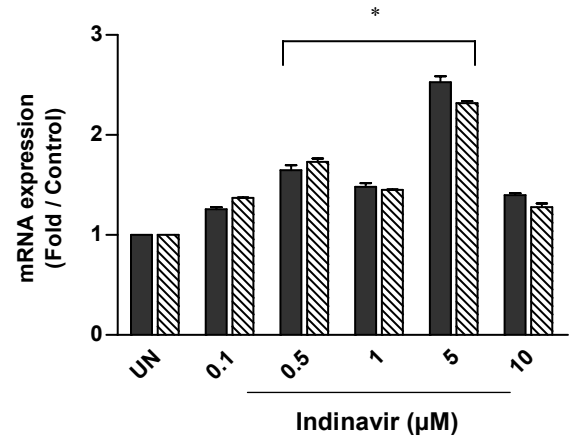

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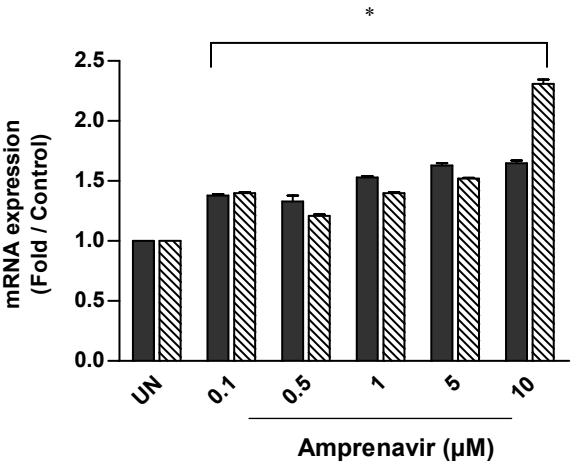


Figure 23. Effect of protease inhibitors on mRNA expression of PXR and CAR

Hepatocytes were treated with A) RTV, B) IDV and C) APV (0 –10 μM) and mRNA expression of PXR (closed bars) and CAR (diagonal thatch bars) was measured. The figure shows the mean of values obtained from four donors, with the S.D indicated by vertical bars. All mRNA values are normalized to β-actin expression. *, significantly different from vehicle treated cells, $p \leq 0.05$

Table 17. Correlation between mRNA expression of transporters and PXR after treatment with protease inhibitors

Drug	Gene	Pearson's CC (r^2)	p-value	Correlation	Spearman's CC (ρ)	p-value	Correlation
RTV	MDR1	0.715	0.035	Yes	1.000	0.0083	Yes
	MRP2	0.971	0.001	Yes	0.9	0.041	Yes
	MRP6	0.694	0.039	Yes	-0.9	0.041	Yes
	BSEP	0.793	0.021	Yes	0.9	0.041	Yes
IDV	MDR1	0.373	0.098	No	0.942	0.0083	Yes
	MRP2	0.621	0.031	Yes	0.942	0.0083	Yes
	MRP6	0.404	0.0875	No	0.257	0.329	No
	BSEP	0.861	0.0038	Yes	0.942	0.0083	Yes
APV	MDR1	0.874	0.0031	Yes	0.885	0.016	Yes
	MRP2	0.965	0.0002	Yes	0.942	0.0083	Yes
	MRP6	0.734	0.0146	Yes	-0.657	0.087	No
	BSEP	0.440	0.075	No	0.6	0.12	No

Table 18. Correlation between mRNA expression of transporters and CAR after treatment with protease inhibitors

Drug	Gene	Pearson's CC (r^2)	p-value	Correlation	Spearman's CC (ρ)	p-value	Correlation
RTV	MDR1	0.074	0.328	No	0.3	0.341	No
	MRP2	0.489	0.094	No	0.4	0.258	No
	MRP6	0.113	0.29	No	-0.1	0.475	No
	BSEP	0.118	0.285	No	0.1	0.475	No
IDV	MDR1	0.443	0.074	No	0.885	0.016	Yes
	MRP2	0.766	0.011	Yes	1.00	0.001	Yes
	MRP6	0.369	0.1	No	0.371	0.248	No
	BSEP	0.958	0.0003	Yes	1.00	0.001	Yes
APV	MDR1	0.502	0.057	No	0.885	0.016	Yes
	MRP2	0.654	0.025	Yes	0.942	0.0083	Yes
	MRP6	0.446	0.073	No	-0.657	0.087	No
	BSEP	0.807	0.007	Yes	0.6	0.120	No

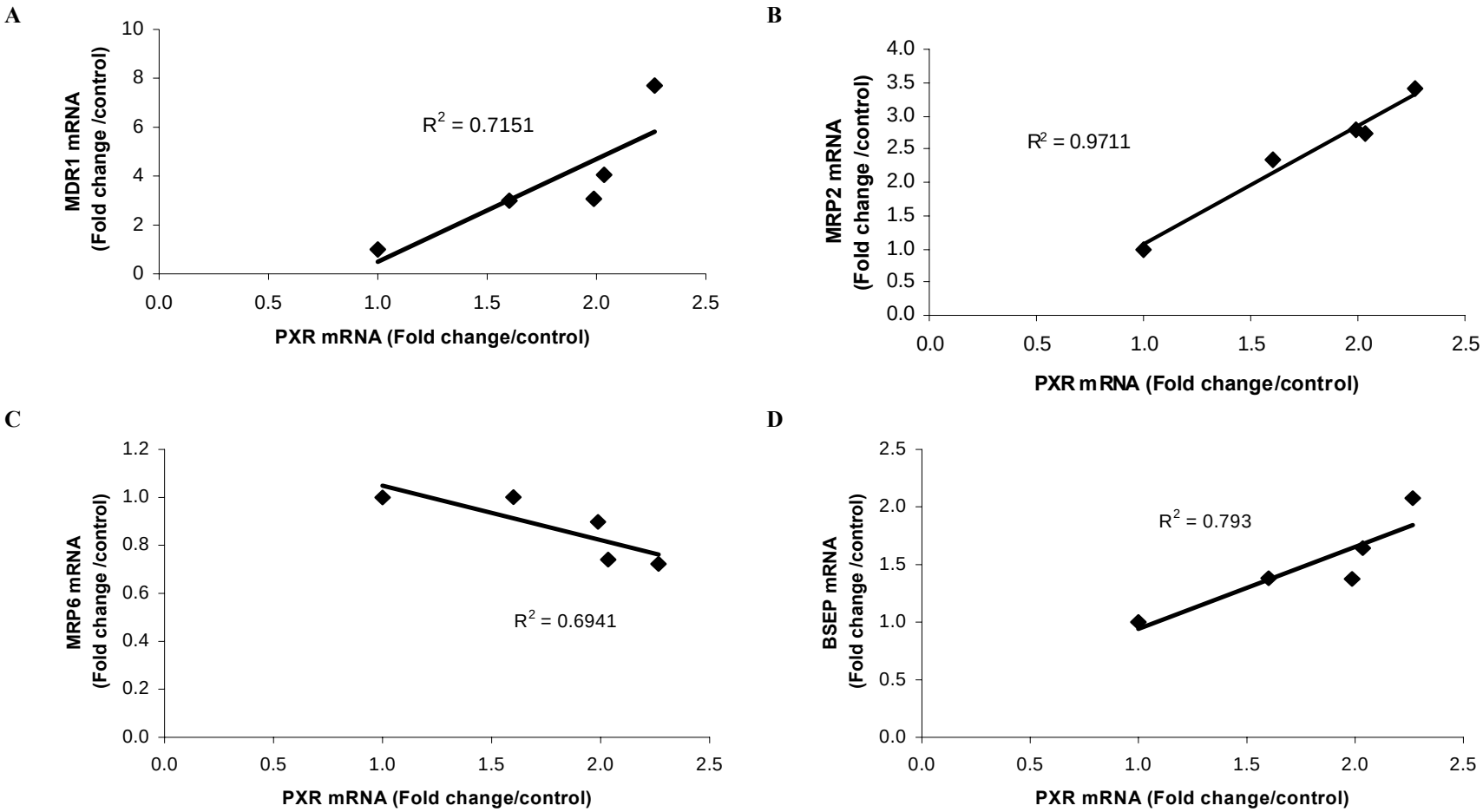


Figure 24. Correlation between transporter mRNA expression and PXR expression after ritonavir treatment

Hepatocytes were treated with RTV (0 –10 μM) and mRNA expression of MDR1, MRP2, MRP6, BSEP and PXR was measured. The figure shows the correlation between PXR mRNA expression and A) MDR1, B) MRP2, C) MRP6 and D) BSEP mRNA. Each value represents the mean of values obtained from four livers. All mRNA values are normalized to β-actin expression. For correlations a p-value of 0.05 was considered statistically significant.

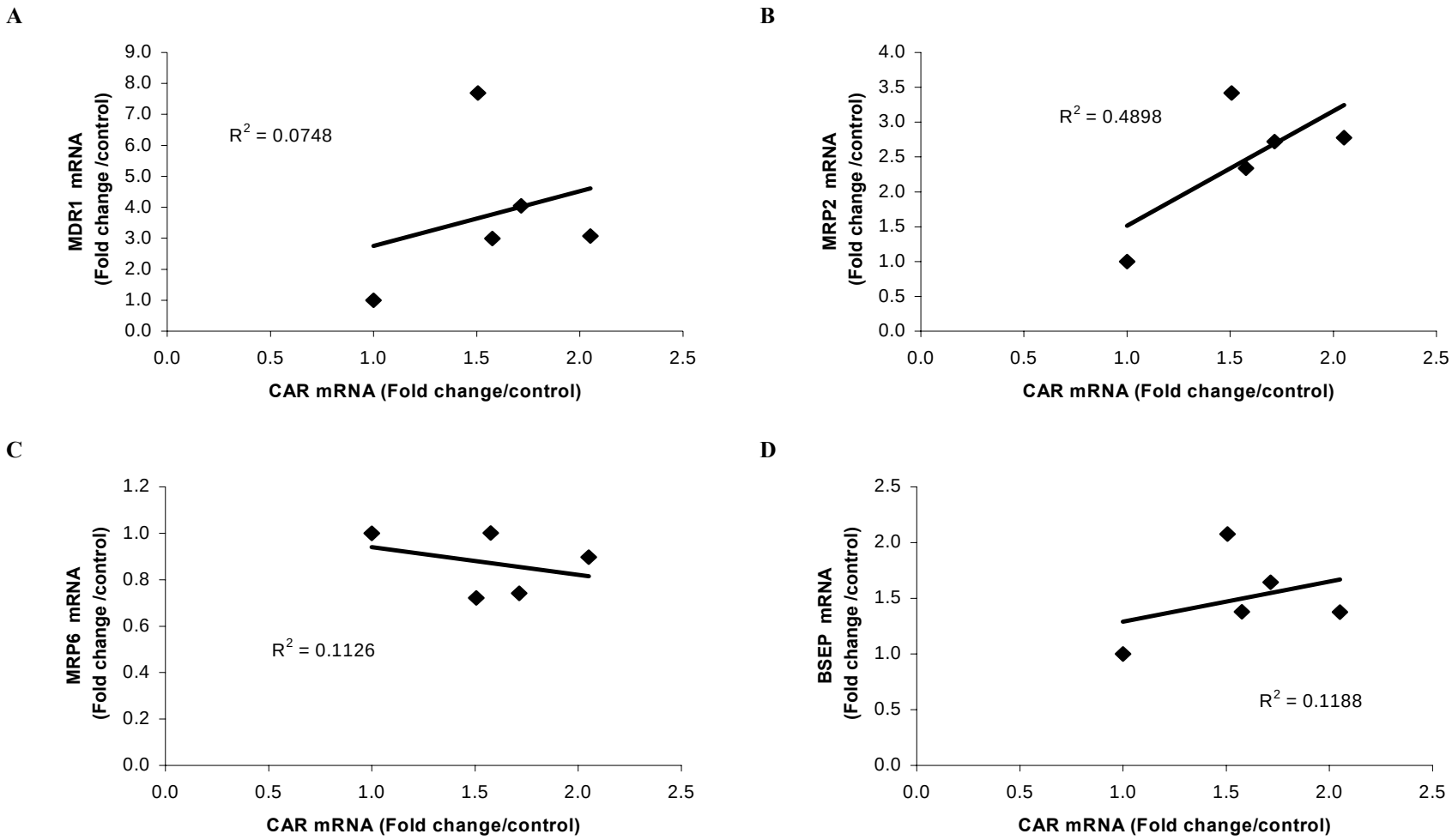


Figure 25. Correlation between transporter mRNA expression and CAR expression after ritonavir treatment

Hepatocytes were treated with RTV (0 –10 μM) and mRNA expression of MDR1, MRP2, MRP6, BSEP and CAR was measured. The figure shows the correlation between CAR mRNA expression and A) MDR1, B) MRP2, C) MRP6 and D) BSEP mRNA. Each value represents the mean of values obtained from four livers. All mRNA values are normalized to β-actin expression. For correlations a p-value of 0.05 was considered statistically significant.

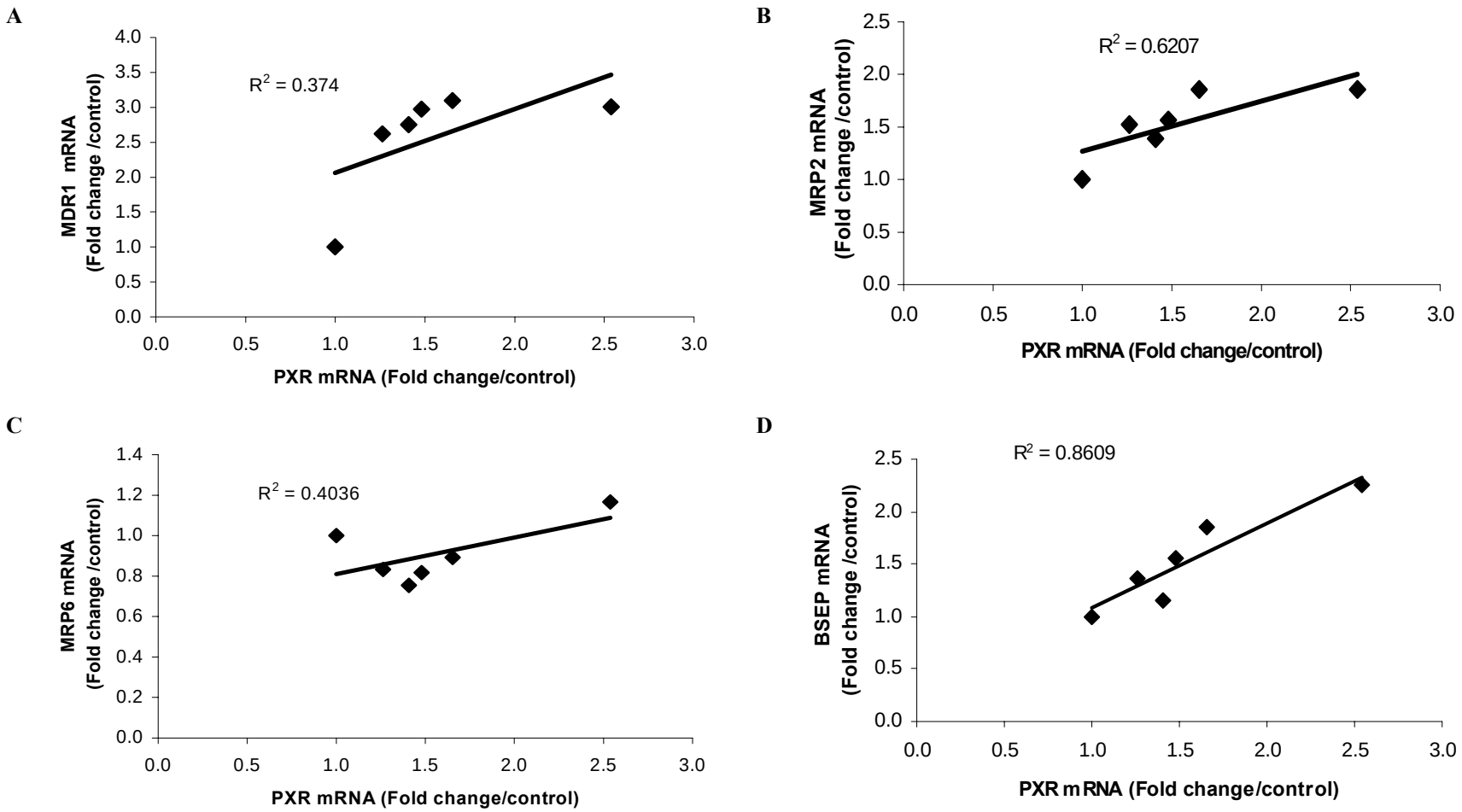


Figure 26. Correlation between transporter mRNA expression and PXR expression after indinavir treatment

Hepatocytes were treated with IDV (0 –10 μM) and mRNA expression of MDR1, MRP2, MRP6, BSEP and PXR was measured. The figure shows the correlation between PXR mRNA expression and A) MDR1, B) MRP2, C) MRP6 and D) BSEP mRNA. Each value represents the mean of values obtained from four livers. All mRNA values are normalized to β-actin expression. For correlations a p-value of 0.05 was considered statistically significant.

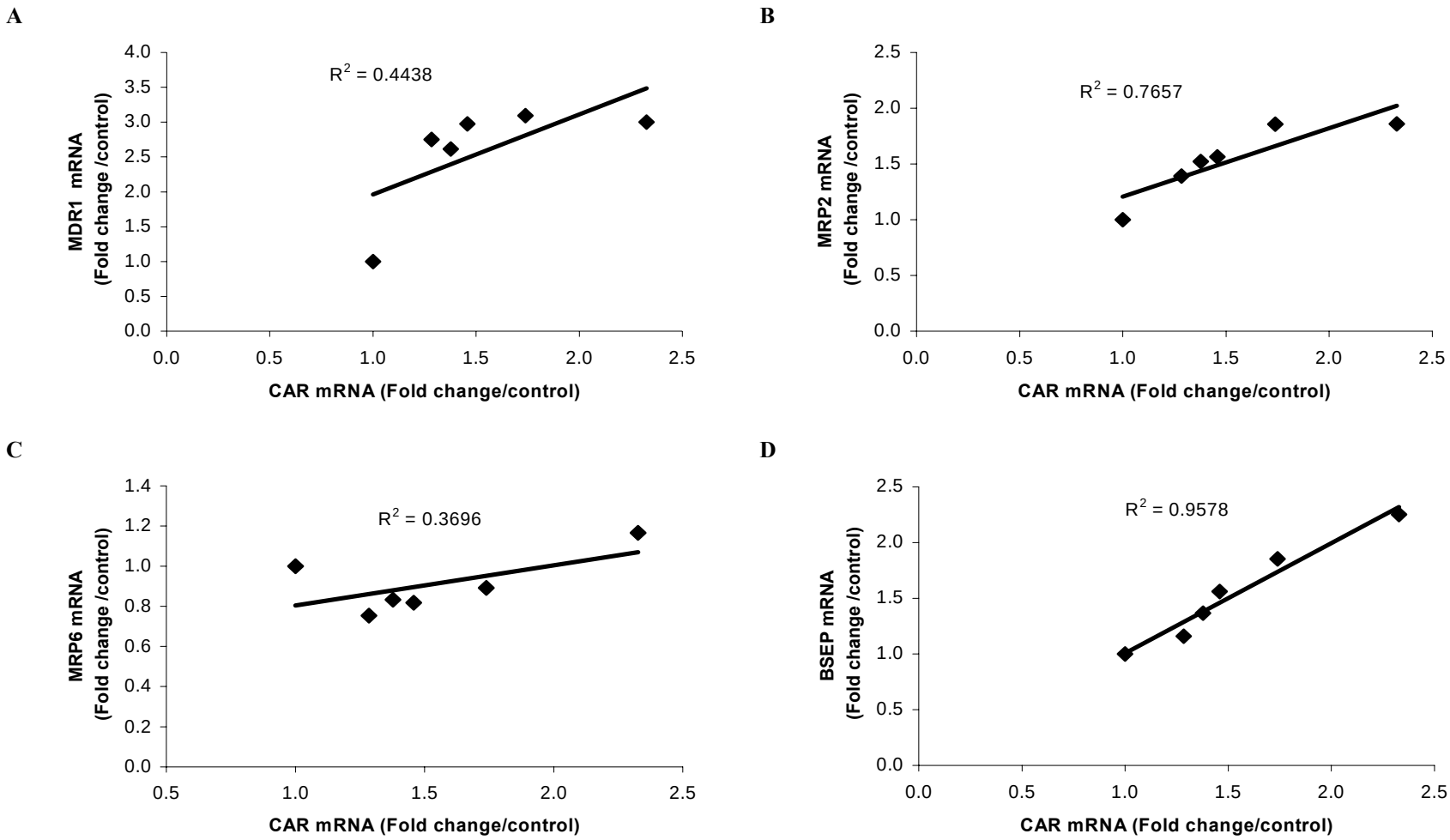


Figure 27. Correlation between transporter mRNA expression and CAR expression after indinavir treatment

Hepatocytes were treated with IDV (0 –10 μM) and mRNA expression of MDR1, MRP2, MRP6, BSEP and CAR was measured. The figure shows the correlation between CAR mRNA expression and A) MDR1, B) MRP2, C) MRP6 and D) BSEP mRNA. Each value represents the mean of values obtained from four livers. All mRNA values are normalized to β-actin expression. For correlations a p-value of 0.05 was considered statistically significant.

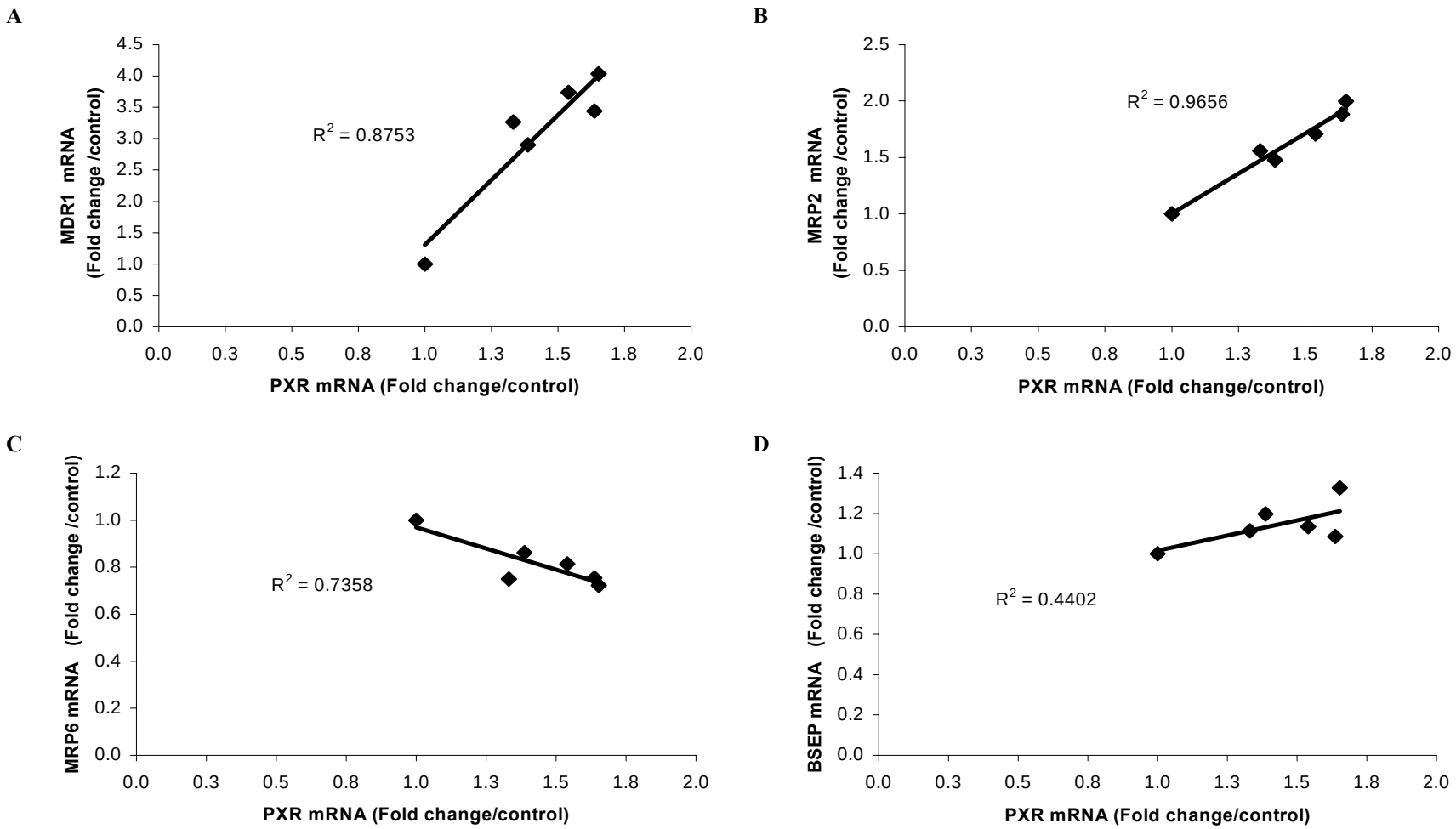


Figure 28. Correlation between transporter mRNA expression and PXR expression after amprenavir treatment

Hepatocytes were treated with APV (0 –10 μM) and mRNA expression of MDR1, MRP2, MRP6, BSEP and PXR was measured. The figure shows the correlation between PXR mRNA expression and A) MDR1, B) MRP2, C) MRP6 and D) BSEP mRNA. Each value represents the mean of values obtained from four livers. All mRNA values are normalized to β-actin expression. For correlations a p-value of 0.05 was considered statistically significant.

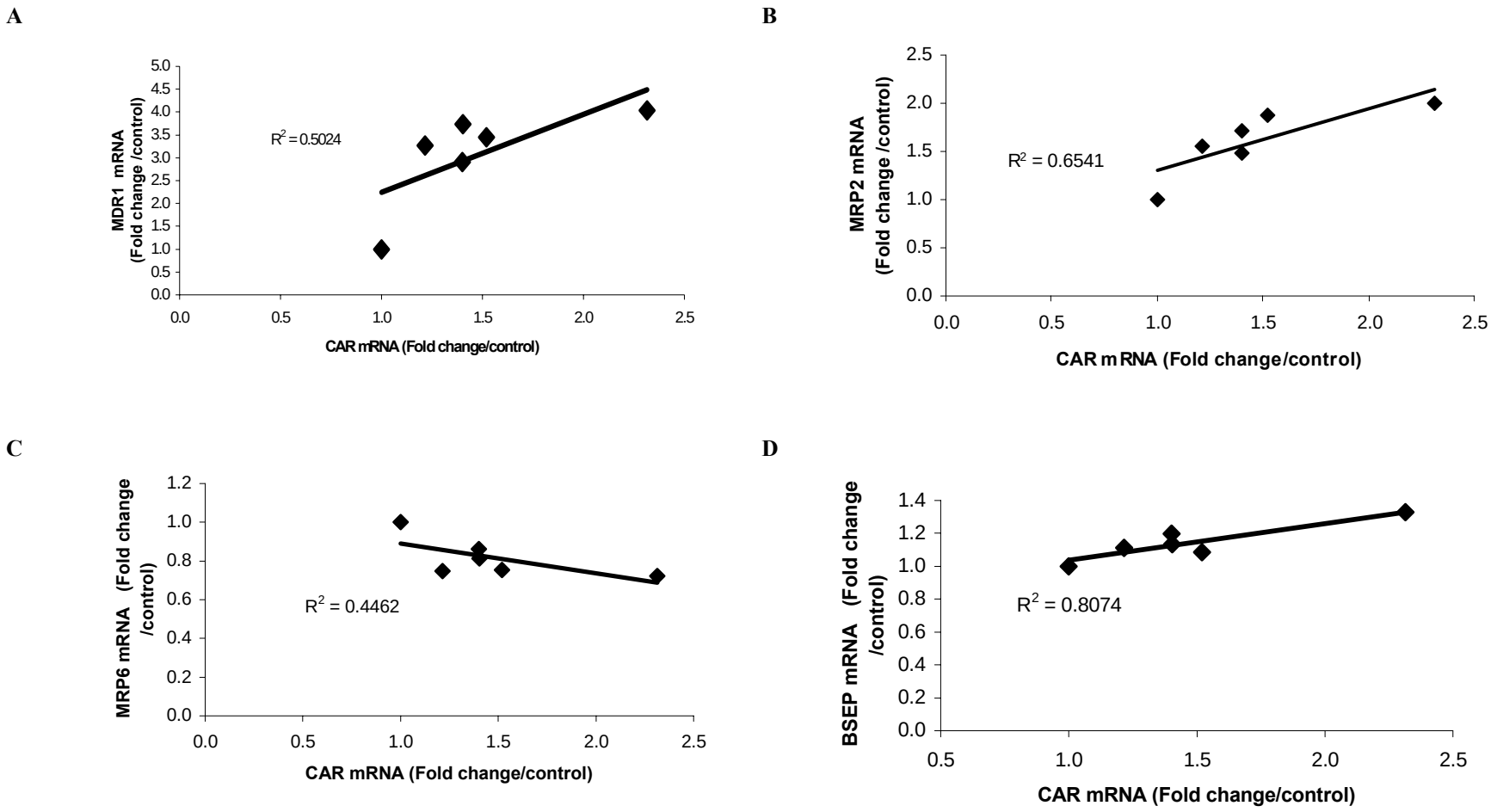


Figure 29. Correlation between transporter mRNA expression and CAR expression after amprenavir treatment

Hepatocytes were treated with IDV (0 –10 μM) and mRNA expression of MDR1, MRP2, MRP6, BSEP and CAR was measured. The figure shows the correlation between CAR mRNA expression and A) MDR1, B) MRP2, C) MRP6 and D) BSEP mRNA. Each value represents the mean of values obtained from four livers. All mRNA values are normalized to β-actin expression. For correlations a p-value of 0.05 was considered statistically significant.

5.6. Discussion

In the present study, we have characterized the effect of protease inhibitors on the expression of drug transporters namely P-glycoprotein, MRP2, MRP6 and BSEP in a human hepatocyte culture system. Human hepatocyte cultures represent an intact and physiologically relevant model useful for studying modulation of hepatic drug transporters. These cells are capable of synthesizing normal bile acids from their cholesterol precursors, formation of their conjugated metabolites, and the canalicular efflux of both parent and metabolite. They also have all the cofactors and various regulatory elements needed to maintain and induce expression of transporter proteins. The application of extracellular 3-D matrix in the form of Matrigel^{TM} prevents the loss of albumin synthesis, leads to phosphorylation of hepatocyte growth factors and epidermal growth factor receptors and results in cuboidal, polar hepatocyte structure (Sidhu and Omiecinski, 1995; Toritsuka et al., 2001; Engl et al., 2004). Previous studies carried out in our laboratory have shown that the basal expression of the transporters such as BSEP, MDR1 and MRP2 is significantly higher in cultures coated with Matrigel^{TM} compared to normal monolayered hepatocyte cultures.

Currently there are very few studies documenting the effect of HIV protease inhibitors on drug transporters. In the present study, we have shown that ritonavir has a differential effect on various hepatic efflux transporters. After RTV treatment, the inductive effect on MDR1 mRNA expression is most pronounced (8.2-fold), followed by that on MRP2 (2.6-fold) and BSEP (2-fold). On the other hand, RTV treatment decreased the expression of MRP6 to 60.6%. The increase in mRNA expression of MDR1 and MRP2 is comparable to that observed after rifampicin (10 μM) treatment. These observations are in agreement with previous studies that

have similarly shown to increase the expression and activity of P-glycoprotein in LS180V cells after RTV treatment (Perloff et al., 2003).

In the current study, indinavir showed a moderate effect on MDR1, MRP2 and BSEP expression with a 2-fold increase in the expression of these transporters, with no effect on MRP6 expression. After amprenavir (10μM) treatment, increase in mRNA expression is highest for MDR1 followed by MRP2 and BSEP with no significant effect on MRP6. There are no previous reports in literature documenting the effect of indinavir and amprenavir on the expression and activity of hepatic efflux transporters.

Earlier studies have shown that ritonavir is a potent PXR activator (Luo et al., 2002). Hepatic efflux transporters such as P-glycoprotein, MRP2 and BSEP are also transcriptionally regulated by the nuclear orphan receptor such as pregnane X receptor (PXR) and constitutive androgen receptor (CAR). After ligand binding in the cytosol, PXR or CAR translocate to the nucleus where they heterodimerize with retinoid X receptor (RXR) and then bind to promoter region on respective transporter gene resulting in increased mRNA expression of corresponding transporter. Therefore, in the present study, correlation analysis was carried out to determine if the effects of protease inhibitors on expression of transporters is mediated through regulation of PXR and/or CAR.

It is shown that after RTV treatment, increase in mRNA expression of MDR1, MRP2 and BSEP correlated well with increased PXR expression, thus suggesting the role of PXR in mRNA induction of the above transporters. On the contrary, no involvement of CAR is observed in modulating the expression of MDR1, MRP2 and BSEP. Similarly, after APV treatment, increase in mRNA expression of MDR1 and MRP2 is potentially mediated mainly through PXR with minimal contribution of CAR. Inconclusive data were obtained for regulation of BSEP after

APV treatment. After IDV treatment, increase in mRNA expression of MRP2 and BSEP is mediated through a mechanism involving both PXR and CAR.

Decrease in mRNA expression of MRP6 after RTV treatment was associated with increased PXR mRNA expression. But, no changes in MRP6 mRNA expression were observed after indinavir and amprenavir treatment. This suggests that possibly other nuclear receptors or transcriptions factors are involved in the regulation of MRP6, which warrants further study.

In summary, this is the first study to report that increasing concentration of HIV-protease inhibitors have differential effects on expression of drug efflux transporters namely P-glycoprotein, MRP2, MRP6 and BSEP in human hepatocyte system. The modulation of transporter expression is possibly mediated via mechanisms involving nuclear receptors such as PXR and/or CAR. Knowing the effects of protease inhibitors on the expression and activity of hepatic transporters will prove highly beneficial in understanding the pharmacokinetic behavior of drugs that are transported via P-glycoprotein, MRP2, MRP6 and BSEP. Given the magnitude of change in transporter activity that is observed in this study, HIV-protease inhibitors are seem to contribute more towards pharmacokinetics interactions with immunosuppressive drugs due to their effect on CYPs and not as much due to their effect on transporters.

6. Effect of pro-inflammatory cytokines on hepatic drug transport in human hepatocytes

6.1. Abbreviations

[3H]TC	[3H] taurocholate
BSEP	bile salt export pump
CAR	constitutive androgen receptor
CsA	cyclosporine A
FXR	farnesoid X receptor
HMM	hepatocyte maintenance medium
IL-1β	interleukin-1β
IL-2	interleukin-2
IL-6	interleukin-6
LPS	lipopolysaccharides
MDR1	multidrug resistant protein 1
MRP2	multidrug resistance associated protein 2
Ntcp	sodium-dependent taurocholate cotransporting protein
PCHH	primary cultures of human hepatocytes
P-gp	P-glycoprotein
PXR	pregnane X receptor
TNFα	Tumor Necrosis Factor-α

6.2. Abstract

Aims: Hepatic canalicular membrane transporters such as P-glycoprotein (P-gp), multidrug resistance protein 2 (MRP2) and bile salt export pump (BSEP) are involved in removal of drugs and endogenous substances from the blood. Immunosuppressive agents are substrates for these transporters. Thus alterations in expression and activity of transporters are though to alter the pharmacokinetics of immunosuppressive agents. Cytokines released during infection have shown to downregulate various hepatic drug transporters in rat *in vivo* and *in vitro* model systems, but very limited data on the regulation of human transporters is available. The objective of the present study was to evaluate the effect of different cytokines on the expression and activity of hepatic drug transporters such as BSEP using primary cultures of human hepatocytes.

Methods: Human hepatocytes in Matrigel^{TM} coated cultures were exposed to IL-1, IL-2, IL-6 and TNFα (0-10 ng/ml) and the effect on BSEP mRNA expression and activity were studied. The mRNA expression was measured using quantitative Real-time PCR. BSEP activity was measured by [3H] taurocholate ([3H] TC) efflux.

Results: Exposure of human hepatocytes to IL-1, IL-2, IL-6 and TNFα resulted in decreased mRNA expression and activity of BSEP, with maximal suppression being observed after 72 hours of treatment. IL-1 decreased BSEP mRNA expression by 80%, while decreasing the BSEP mediated [3H] TC efflux by more than 70%. IL-2 did not significantly alter BSEP mRNA expression and activity, while IL-6 at higher concentration increased BSEP mRNA expression along with decrease in BSEP activity by only 20%. TNFα significantly decreased both, BSEP mRNA expression and activity, by 70 %.

Conclusions: Duration of human hepatocytes to cytokine is an important factor affecting downregulation of BSEP mRNA expression and activity. Different cytokines exhibit different inhibition potential in downregulating BSEP expression and activity which can be ranked as IL-1≅ TNFα > IL-6 >IL-2. Alteration of these cytokines in diseased states such as infection, cancer or organ rejection after transplantation will modulate the expression and activity of drug transporters, thereby impacting the pharmacokinetics of substrate drugs and bile secretion process in liver leading to cholestasis.

6.3. Introduction

Hepatobiliary transport and subsequent bile flow play an important role in the removal of endogenous and exogenous substances from the body (Faber et al., 2003). Hepatic efflux transporters such as P-glycoprotein (P-gp) and multidrug resistance protein 2 (MRP2) are involved in the clearance of drugs used in transplantation such as cyclosporine, tacrolimus and mycophenolic acid, while bile salt export pump (BSEP) is integral in the canalicular handling of conjugated and unconjugated bile salts, such as taurocholate (Gerloff et al., 1998). Alterations in the expression and activity of these transport proteins thus can lead to changes in the pharmacokinetics of drugs transported by these proteins and also can affect the normal physiological processes in the liver.

A number of studies suggest that inflammatory response involving various cytokines such as interleukin-1 (IL-1), interleukin –6 (IL-6) and tumor necrosis factor-α (TNFα) are significantly involved in the downregulation of various hepatic drug transporters (Piquette-Miller et al., 1998; Lee and Piquette-Miller, 2001; Sukhai et al., 2001; Lee and Piquette-Miller, 2003; Cherrington et al., 2004; Siewert et al., 2004). *In vitro* and *in vivo* studies in rats indicate that lipopolysaccharides (LPS), TNFα, IL-1 and IL-6 decrease the uptake and secretion of bile components by altering the expression and activity of hepatic transporters, such as Ntcp, Bsep and Mrp2, thus leading to cholestatic conditions (Green et al., 1994; Whiting et al., 1995; Green et al., 1996; Muhlfeld et al., 2003).

There are very few reports documenting the effect of cytokines on human hepatic transporters. Studies done in human cell lines and in human liver slices have reported downregulation in expression of transporters such as MDR1, MRP2, MRP3 and BSEP after exposure to LPS or individual cytokines and this downregulation is thought to be due to

posttranscriptional changes in transporter gene by cytokines (Elferink et al., 2004; Hisaeda et al., 2004).

Transcriptional factors such as pregnane X receptor (PXR), constitutive androgen receptor (CAR) and farnesoid X receptor (FXR) play an important role in the regulation of drug transporters. Downregulation of these nuclear receptors has been reported after administration of endotoxin in rats. Based upon the above studies, we hypothesized that in human hepatocytes, cytokines will downregulate expression and activity of hepatic efflux transporters and this suppression will be due to alterations in the expression of transcriptional factors, PXR and/or CAR.

In the present study, we have evaluated the effect of increasing concentrations of IL-1β, IL-2, IL-6 and TNFα on the expression and activity of bile salt express pump (BSEP) using human hepatocyte cultures.

6.4. Methods

6.4.1. Evaluation of the cytotoxicity of pro-inflammatory cytokines to human hepatocytes

Hepatocytes in 3D cultures were exposed to individual cytokines IL-1, IL-2, IL-6 and TNFα (0–50 ng/ml) for 72 hours. Following aspiration of media, 10% v/v of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to HMM and incubated for 30 min. At 30 min, the medium was aspirated and cells washed with HMM. Isopropanol (same volume as the medium) was then added and shaken gently for 2 min. Two hundred microliters of this solution was transferred to a 96-well plate, and the absorbance was measured at 570nm.

6.4.2. Hepatocyte treatment protocol to study the effect of pro-inflammatory cytokines on transporters

Twenty-four hours after plating, cells were coated with Matrigel^{TM} (0.233 mg/ml). The effect of time on the expression of various transporters and on BSEP mediated [3H] taurocholate ([3H] TC) efflux was evaluated by exposing the 3D hepatocyte cultures to 5 ng/ml of IL-1, IL-2, IL-6 and TNFα for 24-72 hours. To study the effect of different concentrations of individual cytokines on transporter expression and BSEP activity, hepatocytes were exposed to individual cytokines (0–10 ng/ml) for 72 hours.

Cells were harvested for mRNA by adding 1 mL of Trizol reagent to each well of a 6-well plate. The RNA samples were stored at -20°C for Real Time PCR analysis. Primers for BSEP, PXR, and CAR for the house keeping genes, β-actin and GAPDH were described in Chapter 2. The relative cDNA content was determined from standard curves constructed from serially diluted cDNA samples. The mRNA expression for all genes was normalized to GAPDH in each sample and expressed as fold change over control treatment.

6.4.3. Evaluation of BSEP activity

At the end of the treatment with cytokines, HMM was replaced with Hank's balanced salt solution (HBSS) containing cations (calcium and magnesium) for 20 minutes. After this period, 1 μM [3H]-taurocholate was added in fresh HBSS (with cations) for 20 minutes. Uptake was stopped by aspirating the buffer solution and cells were washed three times with ice cold HBSS (with cations). Fresh HBSS, with and without cations, was then added to the cells for 20 minutes. After this time period, media was sampled and cells harvested in 1 mL of NaOH/SDS

solution. Each sample (0.5 mL) was then counted using a liquid scintillation counter. Aliquots of harvested cells were stored at -80°C for protein determination (Lowry et al., 1951).

6.4.4. Data analysis

All values were calculated as mean ± S.D. For the cytotoxicity analysis (MTT assay), comparisons between treatment groups and untreated control group were made by using a one-way analysis of variance with a post hoc Dunnett's procedure. Data from all other experiments were analyzed using a one-way analysis of variance with a post hoc Tukey's multiple comparison procedure. A p value of ≤ 0.05 was considered statistically significant and all calculations were performed were performed using PRISM software version 4.0 (GraphPad Software, Inc., San Diego, CA, USA).

6.5. Results

Hepatocytes from a total of 12 liver donors were used to conduct the experiments outlined in this Chapter and their relevant demographics, drug history and cell viability can be found in Table 19.

6.5.1. Effect of chronic exposure of hepatocytes to cytokines on MTT reduction

Chronic exposure (72 hours) of human hepatocytes to IL-1, IL-2 and TNFα at concentrations ≥ 20 ng/ml and IL-6 at concentration ≥ 50 ng/ml resulted in significant cellular toxicity as compared to the untreated cells (Figure 30). Thus all the further experiments were carried out with cytokine concentrations at or below 10 ng/ml.

Table 19. Donor information for human hepatocyte preparations used in Chapter 6

Donor HH #	Age	Sex[a]	Race[b]	Cause of death[c]	Drug History	Viability (%)	Percoll separation
1117	68y	F	C	ICH/ stroke	Labetolol, Verapamil, Clonidine	82	No
1118	73y	F	C	HT	Atenolol, Imipramine	80	No
1180	52y	M	C	CVA/ stroke	Dopamine	75	No
1184	66y	F	C	-	None reported	87	Yes
1196	56y	F	C	Asystole	Metoprolol, cholesterol medications (not specified)	79	Yes
1200	53y	F	C	CVA/ stroke	Dopamine	65	Yes
1205	45y	M	H	CVA/ stroke	-	66	No
1209	30y	F	C	CVA/ stroke	Heparin, Ampicillin, Gentamycin, Morphine	66	Yes
1210	35y	F	C	Head trauma	Dopamine, Vasopressin, hydrocortisone	83	Yes
1222	68y	F	C	-	-	93	No
1225	56y	M	C	-	Atenolol, aspirin	77	No
1247							

[a]M, male; F, female; [b]C, Caucasian; H, Hispanic; [c] ICH, intra cranial hemorrhage; CA, cardiac arrest

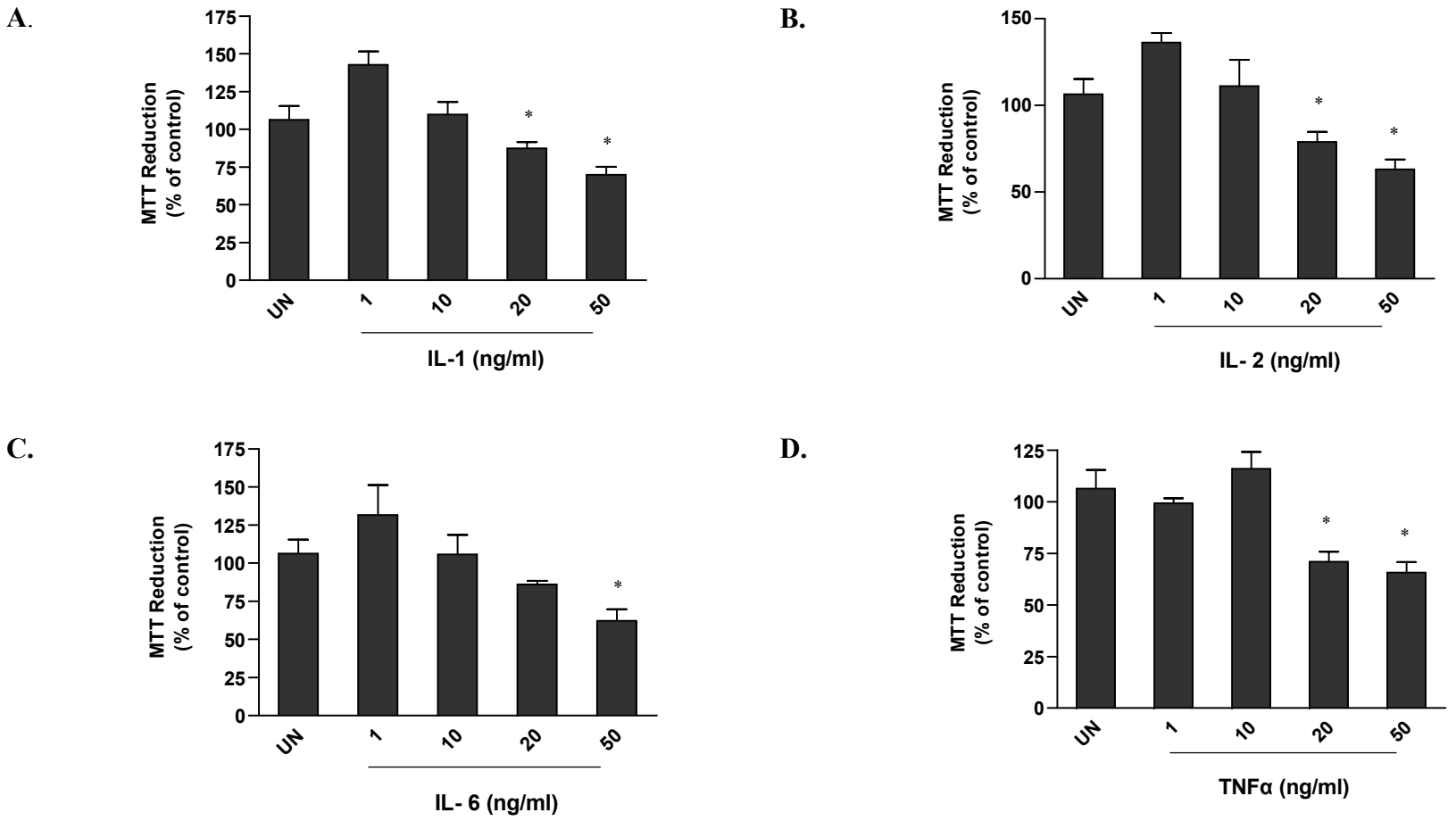


Figure 30. Effect of cytokines on MTT reduction

Human hepatocytes were exposed to A) IL-1, B) IL-2, C) IL-6 and D) TNFα (0-50 ng/ml) for 72 hours in MG overlay cultures and MTT reduction was measured. The figure shows the mean of triplicate treatments from two donors and is expressed as percentage of the value in untreated cells, with S.D. indicated by the vertical bars. *, significantly different from untreated cells, $p \leq 0.05$.

6.5.2. Effect of duration of cytokine exposure on expression and activity of BSEP

To determine the effect of culture times on expression and activity of BSEP, hepatocytes coated with Matrigel^{TM} (0.233 mg/ml) were exposed to each cytokine (IL-1, IL-2, IL-6 and TNFα) at a concentration of 5ng/ml for 24-72 hours. [3H] taurocholate TC ([3H]TC) efflux was used as a measure of BSEP activity. Figure 31A, Figure 32A, Figure 33A and Figure 34A show the effect of IL-1, IL-2, IL-6 and TNFα on the mRNA expression of BSEP, respectively. Maximal suppression of BSEP mRNA expression was observed 72 hours after treating the cells with 5ng/ml of individual cytokines. Similarly, maximal suppression of BSEP mediated [3H]TC efflux was observed 72 hours after treating the cells with 5ng/ml of individual cytokines. BSEP mediated [3H]TC efflux was suppressed to 16.7 ± 5.7 %, 70.6 ± 2.3 % , 79.2 ± 4.1 % and 37.9 ± 0.7 % of control value, after IL-1, IL-2, IL-6 and TNFα treatment, respectively (Figure 31B, Figure 32B, Figure 33B and Figure 34B).

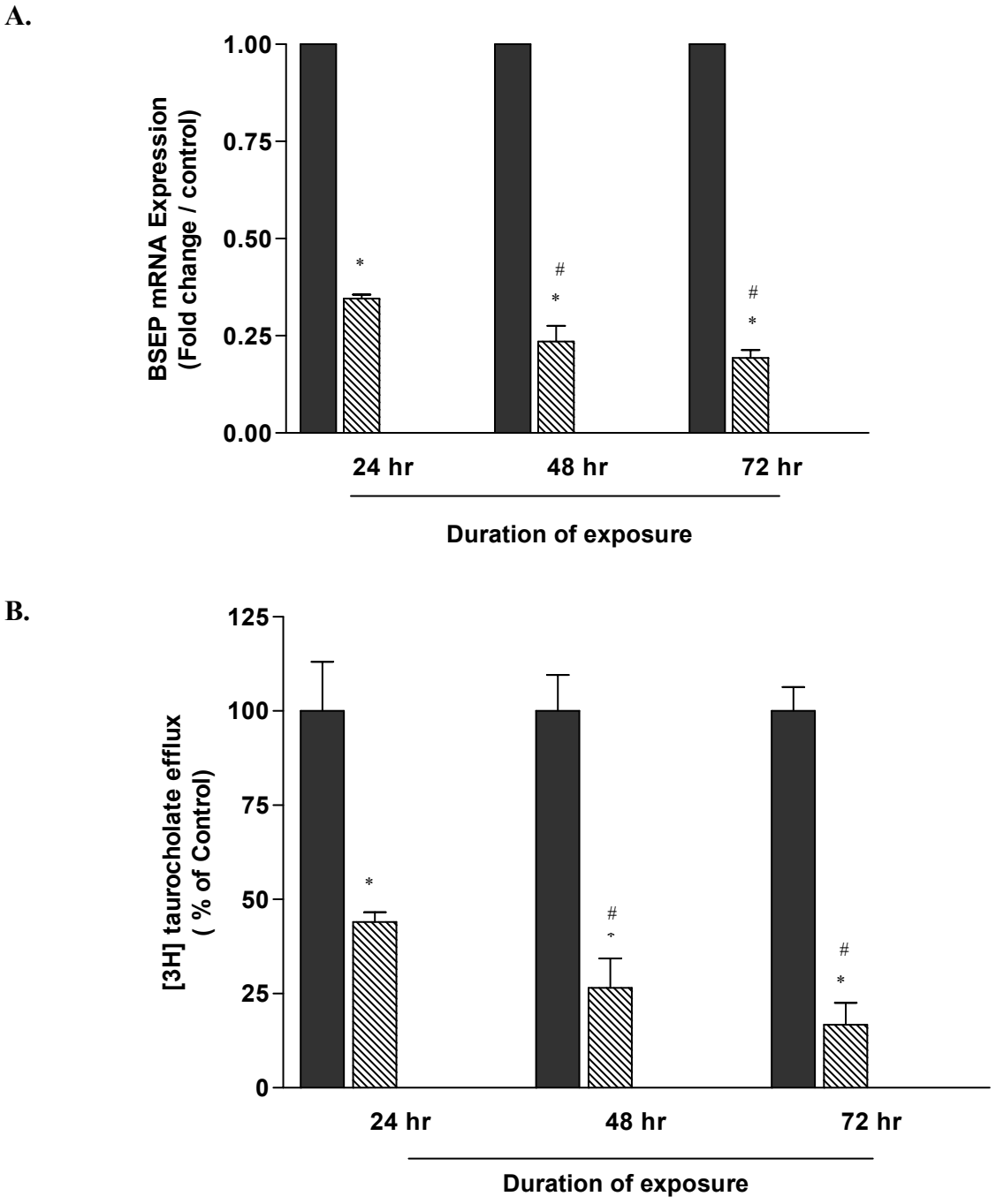


Figure 31. Effect of duration of IL-1 exposure on mRNA expression and activity of BSEP

Hepatocytes were exposed to HMM (closed bars) and 5ng/ml IL-1 (diagonal thatch bars) for 24, 48 and 72 hours in 3D cultures. BSEP A) mRNA expression B) [3H] taurocholate efflux were determined. The figure shows the mean of values obtained from three livers, with S.D. indicated by the vertical bars. All mRNA values are normalized to GAPDH expression. *, significantly different from control; #, significantly different from 24 hour value, $p \leq 0.05$.

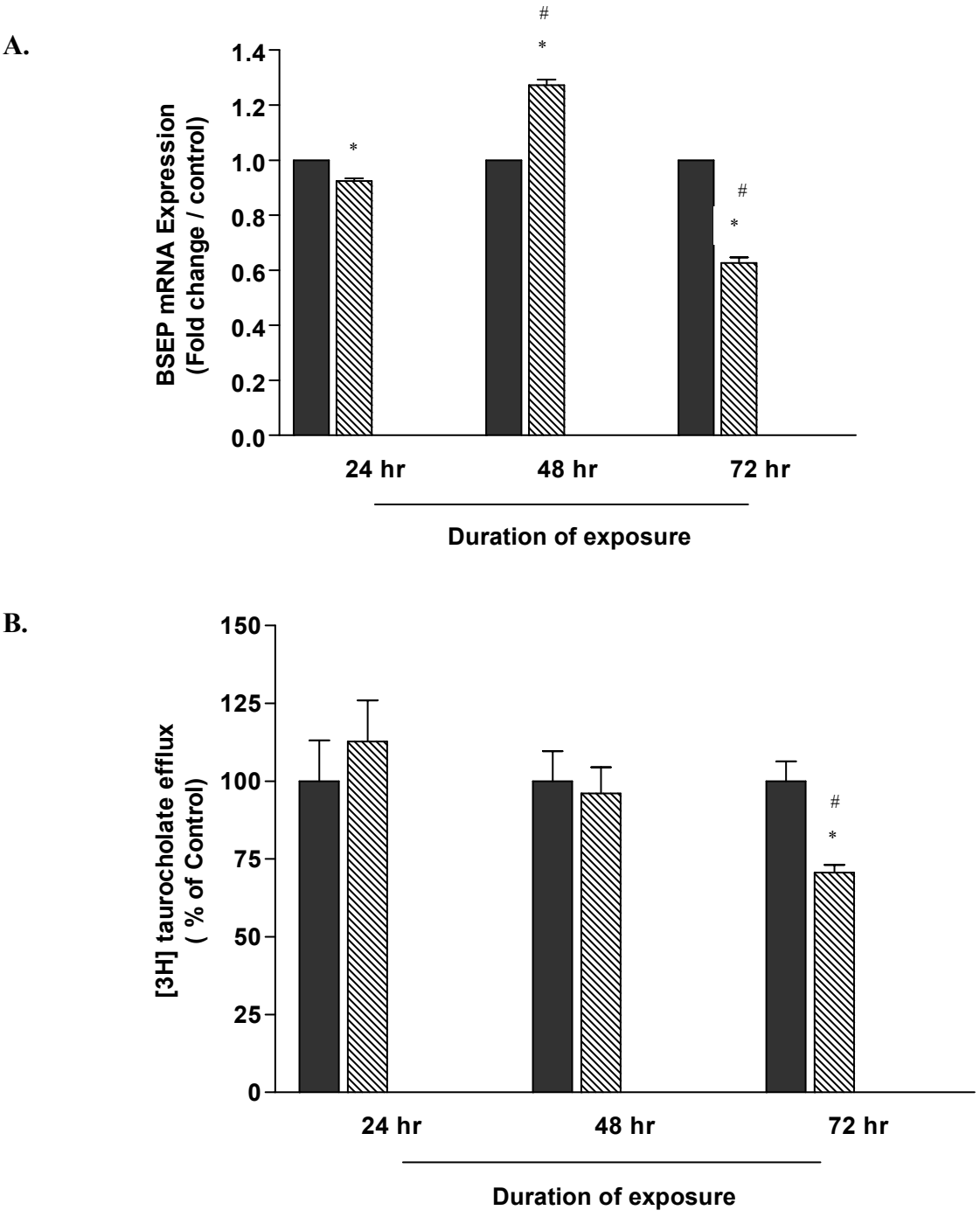


Figure 32. Effect of duration of IL-2 exposure on mRNA expression and activity of BSEP

Hepatocytes were exposed to HMM (closed bars) and 5ng/ml IL-2 (diagonal thatch bars) for 24, 48 and 72 hours in 3D cultures. BSEP A) mRNA expression B) [3H] taurocholate efflux were determined. The figure shows the mean of values obtained from three livers, with S.D. indicated by the vertical bars. All mRNA values are normalized to GAPDH expression. *, significantly different from control; #, significantly different from 24 hour value, $p \leq 0.05$.

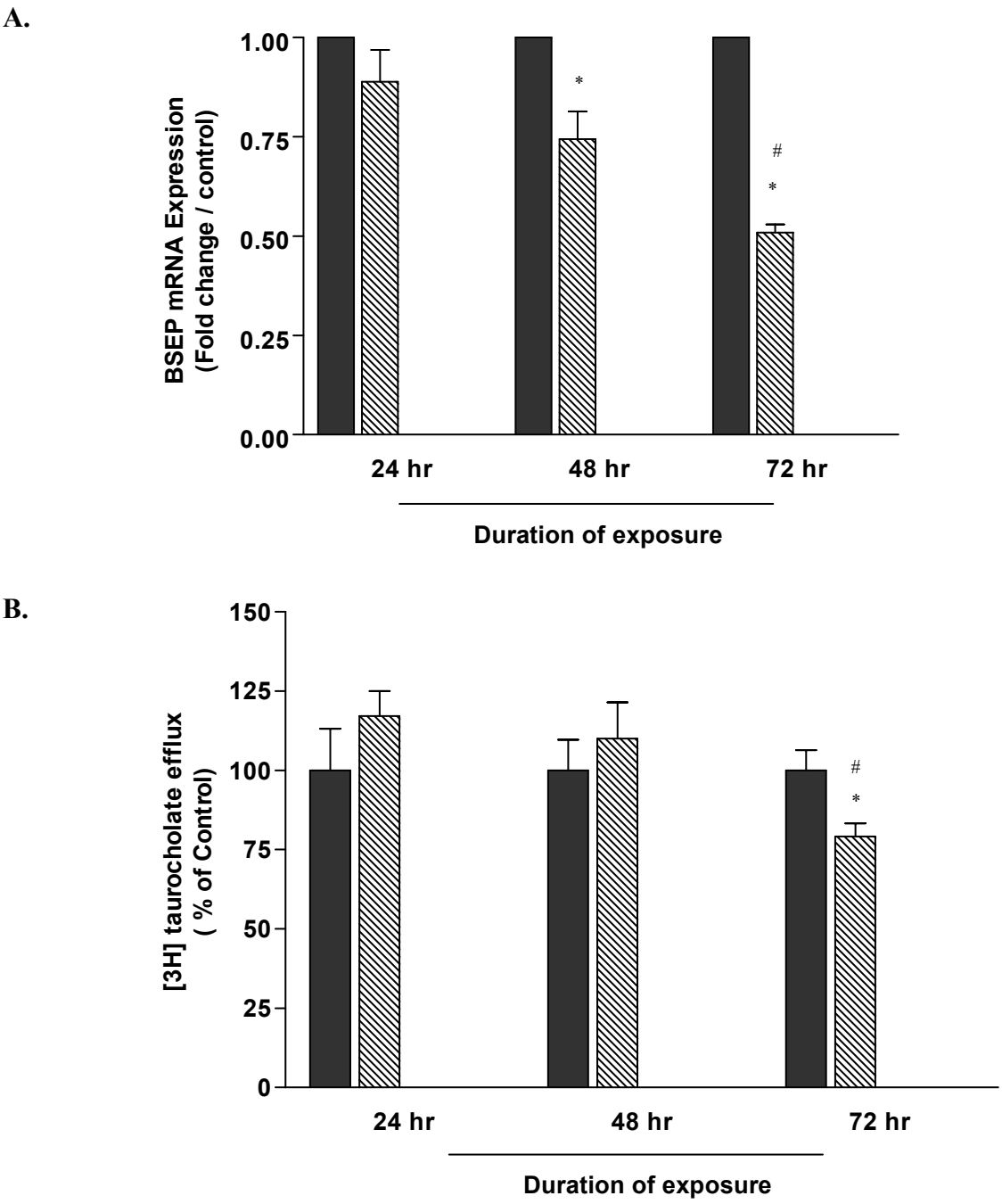


Figure 33. Effect of duration of IL-6 exposure on mRNA expression and activity of BSEP

Hepatocytes were exposed to HMM (closed bars) and 5ng/ml IL-6 (diagonal thatch bars) for 24, 48 and 72 hours in 3D cultures. BSEP A) mRNA expression B) [3H] taurocholate efflux were determined. The figure shows the mean of values obtained from three livers, with S.D. indicated by the vertical bars. All mRNA values are normalized to GAPDH expression. *, significantly different from control; #, significantly different from 24 hour value, $p \leq 0.05$.

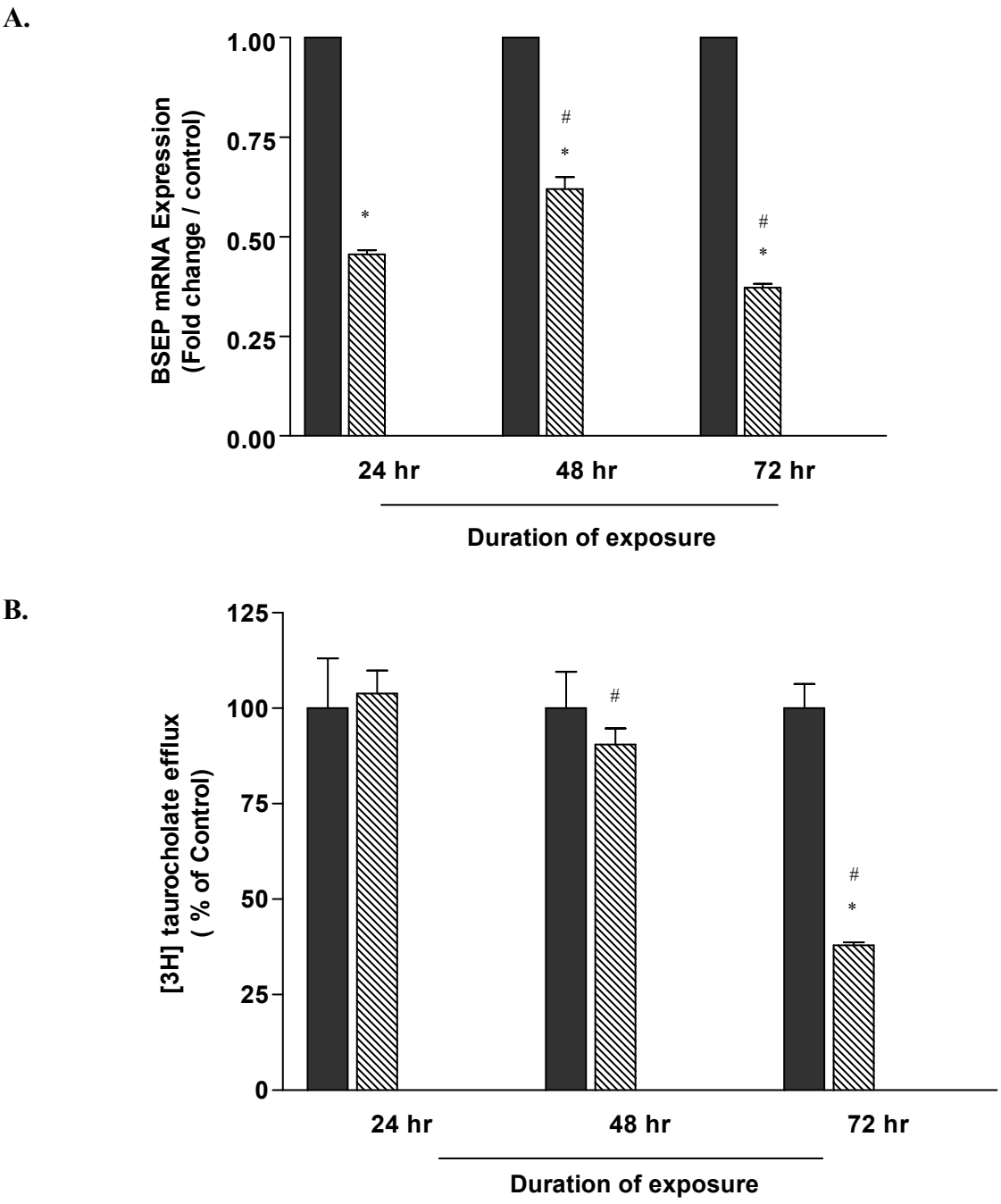


Figure 34. Effect of duration of TNFα exposure on mRNA expression and activity of BSEP

Hepatocytes were exposed to HMM (closed bars) and 5ng/ml TNFα (diagonal thatch bars) for 24, 48 and 72 hours in 3D cultures. BSEP A) mRNA expression B) [3H] taurocholate efflux were determined. The figure shows the mean of values obtained from three livers, with S.D. indicated by the vertical bars. All mRNA values are normalized to GAPDH expression. *, significantly different from control; #, significantly different from 24 hour value, $p \leq 0.05$.

6.5.3. Effect of cytokine concentration on BSEP expression and activity

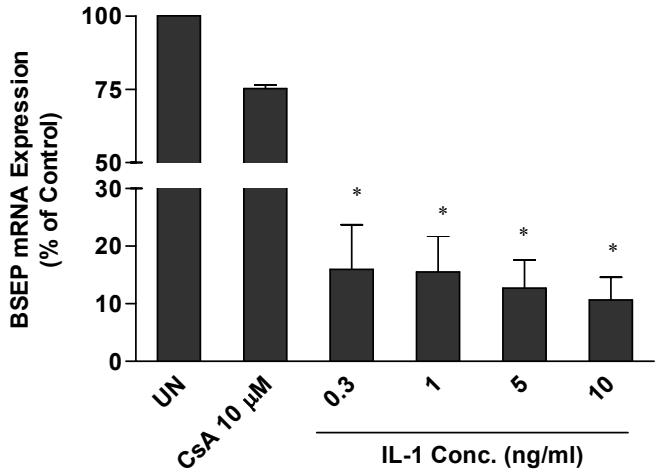
From the previous results, it was found that maximum suppression of BSEP mRNA expression and activity was observed after 72 hours of cytokine treatment. Hence, exposure time of 72 hours was chosen for studying the effect of cytokine concentrations on BSEP expression and activity. Hepatocytes were treated with IL-1, IL-2, IL-6 and TNFα (0-10 ng/ml) for 72 hours. Cyclosporine (CsA) (10 μM), which is an inhibitor of BSEP, was used as a positive control in this study.

Exposure of hepatocytes to IL-1 significantly decreased the mRNA expression of BSEP at all concentrations with a maximum decrease to 10.7 ± 3.95% of control value at 10 ng/ml (Figure 35A). Similarly, exposure to IL-1 resulted in a concentration dependent decrease in [3H]TC efflux values which were reduced to 46.8 ± 15.8 % and 27.03 ± 3.8% of control , at concentrations of 0.3 ng/ml and 10 ng/ml, respectively (Figure 35B).

Treatment with IL-2 did not significantly change the mRNA expression of BSEP at the concentration used. Only after 1 ng/ml IL-2 treatment, BSEP mRNA expression reduced to 53.7 ± 13 % of control (Figure 36A). The efflux of [3H]TC was decreased to 70.15 ± 9.27 % of control value at 10 ng/ml IL-2 (Figure 36B).

IL-6 exposure showed no effect on BSEP mRNA expression (at concentration ≤ 5 ng/ml). At 10 ng/ml of IL-6, BSEP mRNA expression was increased 1.73 ± 0.49-fold over control value (Figure 37A). After IL-6 treatment, maximum decrease in BSEP mediated [3H] TC efflux was seen at 5 ng/ml and was 70.02 ± 1.58 % of control value (Figure 37B).

A.



B.

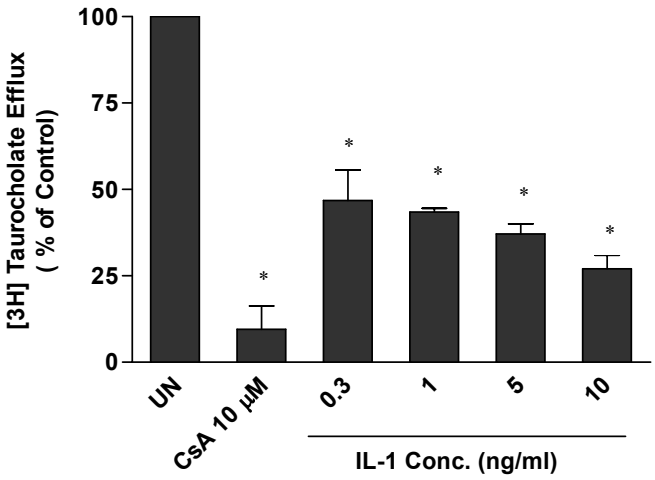
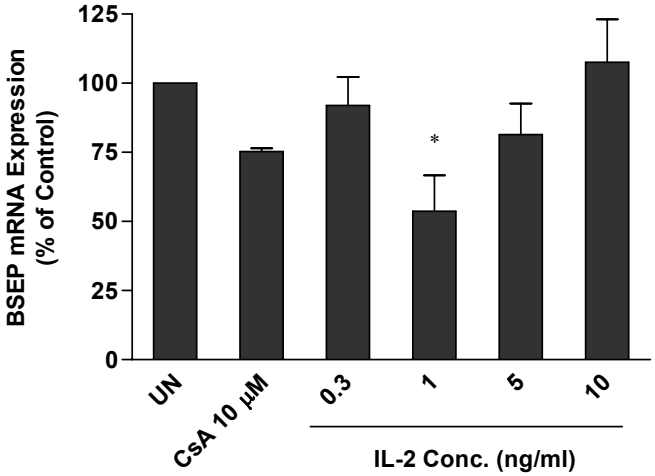


Figure 35. Effect of IL-1 exposure on BSEP mRNA expression and [3H] taurocholate efflux

Human hepatocytes were exposed to IL-1 (0–10 ng/ml). A) BSEP mRNA expression and B) [3H] TC efflux were then determined. The figure shows the mean of values obtained from three livers, with S.D. indicated by the vertical bars. All mRNA values are normalized to GAPDH expression. *, significantly different from untreated cells, $p \leq 0.05$.

A.



B.

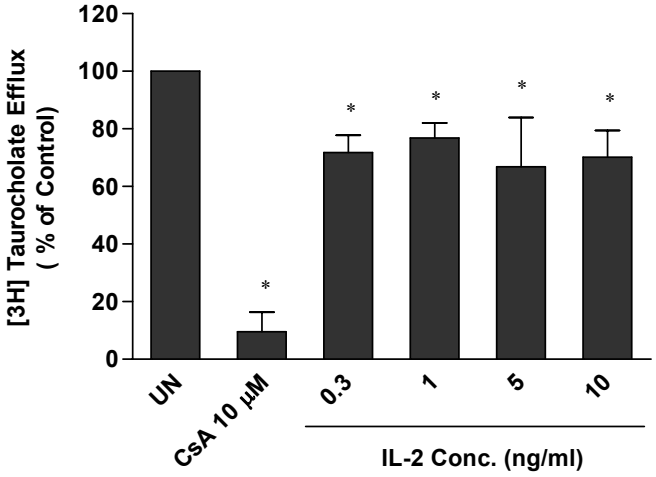
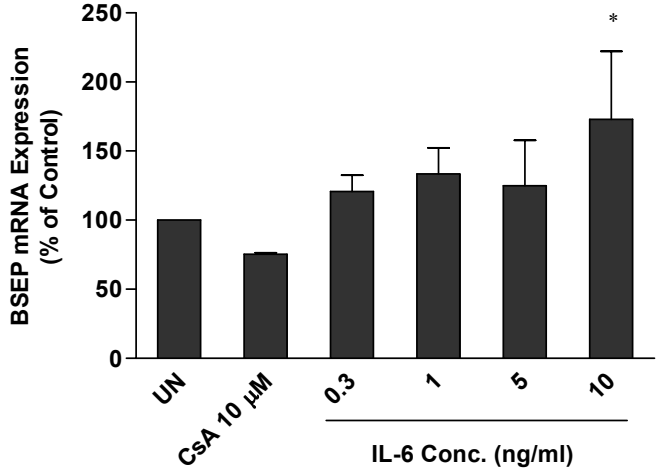


Figure 36. Effect of IL-2 exposure on BSEP mRNA expression and [3H] taurocholate efflux

Human hepatocytes were exposed to IL-2 (0–10 ng/ml). A) BSEP mRNA expression and B) [3H] TC efflux were then determined. The figure shows the mean of values obtained from three livers, with S.D. indicated by the vertical bars. All mRNA values are normalized to GAPDH expression. *, significantly different from untreated cells, $p \leq 0.05$.

A.



B.

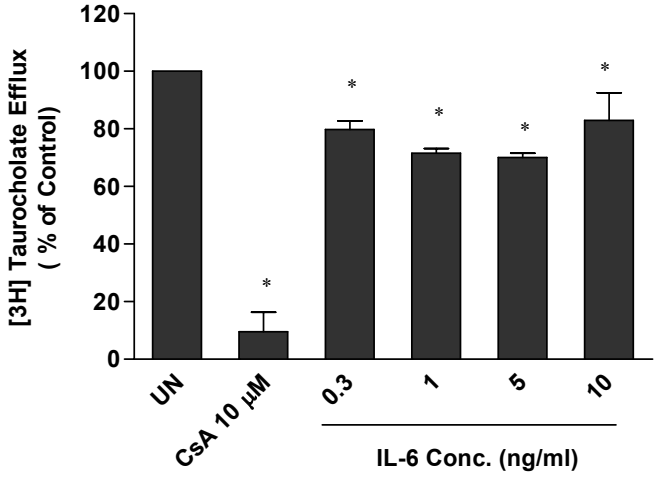


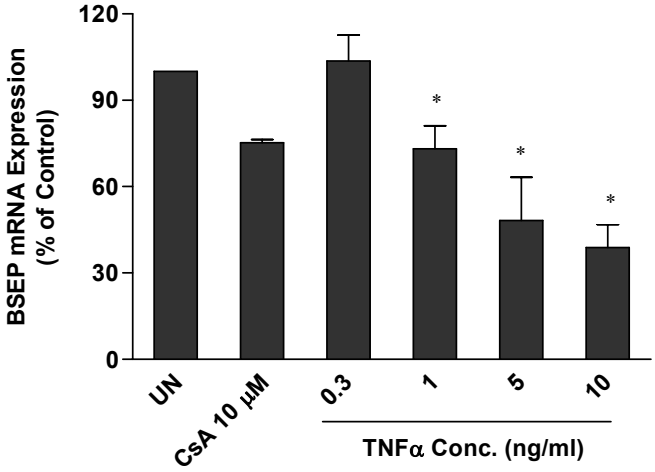
Figure 37. Effect of IL-6 exposure on BSEP mRNA expression and [3H] taurocholate efflux

Human hepatocytes were exposed to IL-6 (0–10 ng/ml). A) BSEP mRNA expression and B) [3H] TC efflux were then determined. The figure shows the mean of values obtained from three livers, with S.D. indicated by the vertical bars. All mRNA values are normalized to GAPDH expression. *, significantly different from untreated cells, $p \leq 0.05$.

Treatment with TNFα showed a significant concentration dependent decrease in both BSEP mRNA expression as well as activity. At TNFα 10 ng/ml, BSEP mRNA expression was decreased to 36.8 ± 8 % (Figure 38A), while [3H] TC efflux was decreased to 5.07 ± 6.3 % of untreated cells (Figure 38B).

Cyclosporine (CsA) (10 μM) reduced BSEP mRNA expression to 75.2 ± 1.1 % and [3H] TC efflux to 9.5 ± 6.7 % of control value.

A.



B.

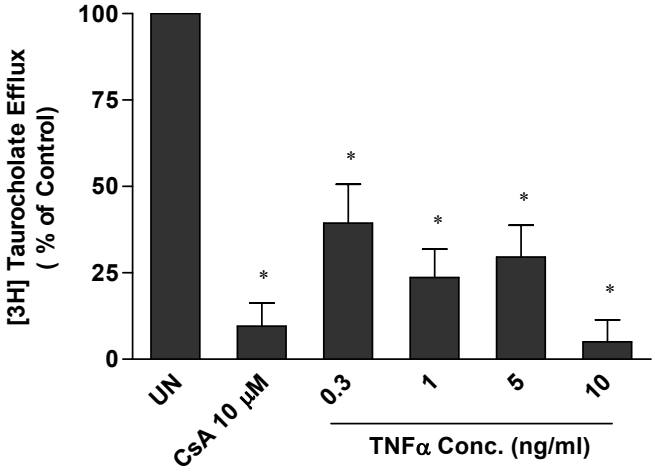


Figure 38. Effect of TNFα exposure on BSEP mRNA expression and [3H] taurocholate efflux

Human hepatocytes were exposed to TNFα (0–10 ng/ml). A) BSEP mRNA expression and B) [3H] TC efflux were then determined. The figure shows the mean of values obtained from three livers, with S.D. indicated by the vertical bars. All mRNA values are normalized to GAPDH expression. *, significantly different from untreated cells, $p \leq 0.05$.

6.6. Discussion

In the present study, we have measured the effect of cytokines (IL-1, IL-2, IL-6 and TNFα) on the mRNA expression and activity of BSEP.

Most of the studies involving use of cytokines are carried out in rodents or using '*in vitro*' systems such as rat hepatocytes or human intestinal or hepatic cell lines. Since different cell types are unique in their ability to produce and release cytokines, it is important to use an appropriate model system to study the effects of cytokines on human hepatic metabolism and transporters.

Modified culture techniques have enabled the use of primary cultures of human hepatocytes to study uptake and efflux of drugs and their metabolites by hepatic drug transporters. Human hepatocytes in culture contain all the necessary cofactors as well as the regulatory elements required to maintain and modulate the transporter proteins and are hence extremely well suited for conducting these studies. Results from preliminary studies conducted in our laboratory and also by others have shown that the basal expression of transporters such as BSEP, MDR1 and MRP2 is significantly higher in cultures coated with Matrigel^{TM} compared to normal monolayered hepatocyte cultures. The loss of tight junctions during the hepatocyte isolation procedure results in a loss of cellular polarity and is believed to affect the expression and activity of some drug transporters by affecting the cellular localization of transporters. The application of an extracellular 3D matrix results in a cuboidal, polar hepatocyte structure and results in a relocalization of transporter proteins such as MDR1, MRP2 and BSEP in hepatic canalicular membrane (Sidhu and Omiecinski, 1995; Toritsuka et al., 2001; Kudryavtseva and Engelhardt, 2003; Engl et al., 2004). For our experiments, Matrigel, which is a mixture of laminin, collagen (IV) heparin sulfate proteoglycans containing growth factors, was chosen as a

matrix because its constituents are thought to more closely mimic those found in human liver (Kleinman et al., 1982; Rodriguez-Antona et al., 2002).

As efflux of taurocholate into bile canaliculi is mainly mediated by bile salt export pump, [3H] Taurocholate ([3H] TC) was used to measure BSEP activity. After treatment with cytokines, [3H] TC efflux was measured in 3D cultures of hepatocytes in absence and presence of the cations containing calcium and magnesium. Only those cells exposed to cation containing medium retain their tight junctions that allow for the formation of canalicular structure (Kostrubsky et al., 2003). [3H] TC, in cells without cations (1mM EDTA is added to medium to chelate residual cation), accounts for all the processes (passive leakage + active transport) by which [3H] TC enter the media. Efflux measured in cells exposed to cation containing medium, reflects only the passive leakage of [3H] TC from the cells. Thus, the difference in these two values (with and without cations) will account for the BSEP mediated transport of [3H] TC into the canalicular spaces, which has been used as an indirect measure of BSEP activity.

In the present study, we have shown that the duration of cytokine exposure to human hepatocytes is an important factor affecting downregulation of BSEP expression and activity. In human hepatocyte system, exposure to all cytokines studied in this work showed maximum downregulation of expression as well as BSEP mediated efflux of [3H]TC after 72 hours exposure. These observations are consistent with another study, where, in human hepatocytes IL-1 and IL-6 are shown to downregulate CYP3A4 mediated testosterone metabolism, with the maximum suppression being seen at 72 hours (Sunman et al., 2004).

Among all the cytokines studied in this work, IL-1 is shown to be most effective in downregulating the mRNA expression and activity of BSEP leading to ~85-90% inhibition in mRNA expression and ~50-75% decrease in BSEP activity. Although there are no reports

showing direct effect of IL-1 on BSEP mRNA expression and activity in human *in vitro* systems, LPS (100 μg/ml) treatment in human liver slices has shown to downregulate BSEP protein content with no changes in mRNA expression (Elferink et al., 2004). In general, IL-1 is shown to downregulate the expression of rodent hepatic efflux transporters such as Mrp2 and Mdr1a and Bsep (Hartmann et al., 2002). Additionally, in HepG2 cells, IL-1 is shown to decrease expression and activity of MRP2 and MDR1 (Hisaeda et al., 2004). The above cited literature findings regarding decreased expression of transporters by IL-1 seem to be consistent with our observations of reduced BSEP expression and activity.

IL-2 did not show any significant decrease in mRNA expression, while showing only ~20-30% decrease in BSEP activity. Sunman et al. (2004) have documented failure of IL-2 in decreasing CYP3A4 mediated metabolism of testosterone in human hepatocyte cultures. However, in the same study, it was shown that in hepatocyte/ Kupffer cell co-cultures, after IL-2 treatment, CYP3A4 activity was decreased by 50-70%. This suggests that IL-2 inhibits CYP3A4 activity 'indirectly' through Kupffer cells by mediating the release of IL-1, IL-6 or TNFα, which are responsible for downregulating CYP3A4 activity. Failure of IL-2 to show any significant suppression of BSEP in our experiments may be attributed to the absence of Kupffer cells in the model system used.

Unlike the behavior of other cytokines used, IL-6 treatment resulted in 1.74-fold increase in BSEP mRNA expression at 10 ng/ml, while showing a decrease of 20-30% in BSEP activity. Contrary to these findings, IL-6 has been shown to significantly decrease the mRNA expression of Bsep by 50-75 % in rat hepatocytes. Thus, the response shown by IL-6 appears to vary in different species with more significant suppression seen in rat hepatocyte compared to human

hepatocytes. Other *In vitro* studies in rat hepatocytes have shown that IL-6 treatment causes a similar decrease in the expression and activity of P-glycoprotein (Sukhai et al., 2001).

TNFα exhibits a concentration dependent decrease in mRNA expression with 70% inhibition at the highest concentration (10 ng/ml) with 50-85% inhibition of BSEP activity. These findings are in agreement with earlier studies in HepG2 cells, which showed decreased MRP2 and MDR1 expression and activity with TNFα at 1-20 ng/ml (Hisaeda et al., 2004). On the other hand, in mice studies, TNFα did not significantly affect Bsep and Mrp2 expression (Hartmann et al., 2002). Cyclosporine (CsA) is a nonspecific inhibitor of BSEP. CsA does not inhibit mRNA expression of BSEP but decreases activity significantly. On the other hand, cytokines decrease BSEP mRNA expression as well as activity.

In summary, it has been shown that in human hepatocytes, maximum suppression in mRNA expression and BSEP activity occurs after 72 hours of cytokine treatment. Different cytokines exhibit different inhibition potential which can be ranked in decreasing order of inhibition as IL-1 ≅ TNFα > IL-6 > IL-2. Alteration of these cytokines in diseased states such as infection, cancer or rejection after transplantation, will modulate the expression and activity of hepatic BSEP, thus impacting the pharmacokinetics of substrate drugs and/or bile secretion leading to cholestasis. Response to cytokines varies between species and caution must be examined in extrapolating studies done in animals to humans. With cytokine studies, co-culture of hepatocytes with Kupffer cells may provide a better system in simulating *in vivo* condition.

7. Effect of pro-inflammatory cytokines on phase II metabolizing enzyme, UGT1A1, in human hepatocytes

7.1. Abbreviations

CAR	constitutive androgen receptor
E3G	estradiol-3-glucuronide
FXR	farnesoid X receptor
HMM	hepatocyte maintenance medium
HNF	hepatocyte nuclear factors
IL-1β	interleukin -1β
IL-2	interleukin -2
IL-6	interleukine-6
LPS	lipopolysaccharides
PB	phenobarbital
PCHH	primary cultures of human hepatocytes
PXR	pregnane X receptor
TNFα	tumor Necrosis Factor-α
UGT	UDP- glucuronosyl transferases
UN	untreated cells

7.2. Abstract

Aims: Hepatic phase II enzymes, UGTs, play an important role in drug disposition and clearance of drugs used in transplant patients, such as mycophenolic acid, acetaminophen and morphine. UGT1A1 is an important isoform involved in the glucuronidation of several drugs as well as endogenous compounds such as bilirubin, estradiol and testosterone. In rat and pig hepatocytes, cytokines are shown to downregulate expression and activity of UGTs while in human liver biopsies, decreased UGT1A4, UGT2B4 and UGT2B7 mRNA levels were observed with increased inflammation. Based on these literature reports, the current study was undertaken to study the effect of different cytokines on expression and activity of hepatic UGT1A1 enzyme, using primary cultures of human hepatocytes. Additionally, the effect of cytokines on mRNA expression of nuclear receptors, namely PXR and CAR that are involved in UGT1A1 regulation was also studied.

Methods: Human hepatocytes in Matrigel^{TM} coated cultures were exposed to IL-1, IL-2, IL-6 and TNFα (0-10 ng/ml) and effect on mRNA expression of UGT1A1, PXR and CAR was determined using quantitative Real-time PCR. UGT1A1 protein was determined using western blotting, while UGT1A1 activity was determined by using estradiol as a probe substance.

Results: Increasing concentrations of IL-1 (0-10 ng/ml) significantly inhibited UGT1A1 mRNA expression, protein content as well as activity with maximal inhibition to 55 %, 47 % and 41 % of control, respectively, at 10 ng/ml. The mRNA expression of PXR and CAR was also decreased to 50–55 % after IL-1 treatment. IL-2 showed slight increase in UGT1A1 mRNA expression with no significant effect on protein content, while UGT1A1 activity was decreased to 80 % of control. IL-2 suppressed PXR and CAR expression only at the highest concentration. IL-6 at higher concentrations showed increase in UGT1A1 mRNA, but protein content and activity were decreased. The mRNA expression of PXR and CAR were also increased at high concentrations of IL-6. Treatment with TNFα resulted in decreased UGT1A1 protein content (54 %) and activity (75%), with no changes in mRNA expression.

Conclusions: Cytokine treatment in general resulted in suppressed the expression and activity of UGT1A1. Different cytokines exhibit different inhibition potential in downregulating UGT1A1 activity and the rank order of potency was IL-1 >IL-6 ≅ TNFα > IL-2, with maximal

inhibition seen after IL-1 exposure. The lowered UGT1A1 activity can affect conjugation of bilirubin during inflammation process along with decreasing the metabolism of drugs conjugated by UGT1A1.

7.3. Introduction

Hepatic phase II metabolizing enzymes, such as UDP- glucuronosyltransferases (UGTs), play an important role in drug disposition and clearance, along with phase I enzymes (CYPs) and various drug transporters. Many drugs used in transplant patients, such as mycophenolic acid; acetaminophen and morphine are mainly metabolized by glucuronidation. In addition, several endogenous compounds such as bilirubin, estradiol and testosterone also undergo glucuronidation.

The regulation of CYP enzymes in models of infection and inflammatory diseases has been studied extensively. The activity and expression of most of the CYP enzymes is shown to be downregulated by pro-inflammatory cytokines. Downregulation of CYP activities or protein levels during inflammation are also generally accompanied by a decrease in the respective CYP mRNA expression (Morgan, 1997; Morgan, 2001). Thus it is suggested that transcriptional suppression could be the primary mechanism for the decrease in CYP mRNA expression during inflammation. Downregulation of transcription factors such as hepatocyte nuclear factors (HNFs), NF-κB along with several nuclear receptors such as pregnane X receptor (PXR) and constitutive androgen receptor (CAR) has been proposed to be responsible for the suppression of CYPs by inflammatory stimuli (Iber et al., 2000; Ke et al., 2001; Beigneux et al., 2002). There is relatively little information about regulation of phase II enzymes during inflammation. However, it is known that PXR and CAR also regulate Phase II enzymes.

Thus, we hypothesized that pro-inflammatory cytokines will downregulate expression and activity of hepatic UGT1A1 enzyme and this suppression will be partly due to alterations in the expression of transcription factors, PXR and/or CAR.

In the present study we have evaluated the effect of increasing concentrations of IL-1β, IL-2, IL-6 and TNFα on the expression and activity of UGT1A1 using primary cultures of human hepatocytes.

7.4. Methods

7.4.1. Evaluation of the cytotoxicity of cytokines to human hepatocytes

Hepatocytes in 3D cultures were exposed to individual cytokines IL-1β, IL-2, IL-6 and TNFα (0–50 ng/ml) for 72 hours. Following aspiration of media, 10% v/v of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to HMM and incubated for 30 min. At 30 min, the medium was aspirated and cells washed with HMM. Isopropanol (same volume as the medium) was then added and shaken gently for 2 min. Two hundred microliters of this solution was transferred to a 96-well plate, and the absorbance was measured at 570nm.

7.4.2. Hepatocyte treatment protocol to study the effect of individual cytokines on UGT1A1 expression and activity

Twenty-four hours after plating, cells were coated with Matrigel^{TM} (0.233 mg/ml). Cells were then exposed to IL-1β, IL-2, IL-6 and TNFα (0, 0.3, 1, 5 and 10 ng/ml) for 72 hours. On the day of study, cells were washed with 1.5 ml of fresh medium for 1 h and then incubated in 1.5 ml of medium containing 250 μM estradiol for an additional 60 minutes. At the end of that time, cell lysate along with the medium was sampled and stored at -80°C for estradiol-3-glucuronide (E-3-G) determination by HPLC. Cells were harvested in phosphate buffer (0.1 M, pH 7.4) and stored at -80°C for protein determination (Lowry O, 1951) and detection of immunoreactive

UGT1A1 protein. The relative amounts of proteins were assessed by the intensity of immunoblot staining carried out by densitometry (ImageJ, v1.34, http://rsb.info.nih.gov.ij)

Cells were also harvested for mRNA by adding 1 mL of Trizol reagent to each well of a 6-well plate. The RNA samples were stored at -20°C for Real Time PCR analysis. Primers for UGT1A1, PXR, CAR and GAPDH were described in Chapter 2. The relative cDNA content was determined from standard curves constructed from serially diluted cDNA samples. The mRNA expression for all genes was normalized to GAPDH in each sample and expressed as fold change over control treatment.

7.4.3. Data analysis

All values were calculated as mean ± S.D. Comparisons among various treatment groups were carried out using a one-way analysis of variance with a post hoc Tukey's multiple comparison procedure. A p value of ≤ 0.05 was considered statistically significant and all calculations were performed were performed using PRISM software version 4.0 (GraphPad Software, Inc., San Diego, CA, USA).

Table 20. Donor information for human hepatocyte preparations used in Chapter 7

Donor HH #	Age	Sex[a]	Race[b]	Cause of death[c]	Drug History	Viability (%)	Percoll separation
1117	68y	F	C	ICH/ stroke	Labetolol, Verapamil, Clonidine	82	No
1118	73y	F	C	HT	Atenolol, Imipramine	80	No
1180	52y	M	C	CVA/ stroke	Dopamine	75	No
1184	66y	F	C	-	None reported	87	Yes
1196	56y	F	C	Asystole	Metoprolol, cholesterol medications (not specified)	79	Yes
1200	53y	F	C	CVA/ stroke	Dopamine	65	Yes
1205	45y	M	H	CVA/ stroke	-	66	No
1209	30y	F	C	CVA/ stroke	Heparin, Ampicillin, Gentamycin, Morphine	66	Yes

[a]M, male; F, female; [b]C, Caucasian; H, Hispanic; [c] ICH, intra cranial hemorrhage; CA, cardiac arrest

7.5. Results

Hepatocytes from a total of 8 liver donors were used to conduct the experiments outlined in this Chapter and their relevant demographics, drug history and cell viability can be found in Table 20.

Chronic exposure (72 hours) of human hepatocytes to IL-1, IL-2 and TNFα at concentrations ≥ 20 ng/mL and IL-6 at concentration ≥ 50 ng/ml resulted in significant cellular

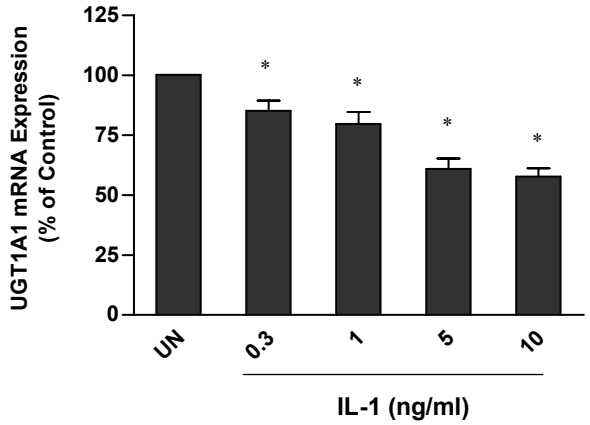
toxicity as compared to the untreated cells (Figure 30, Chapter 6). Thus, these experiments were carried out using cytokine concentrations at or below 10 ng/ml.

7.5.1. Effect of cytokines on UGT1A1 mRNA expression, protein content and activity

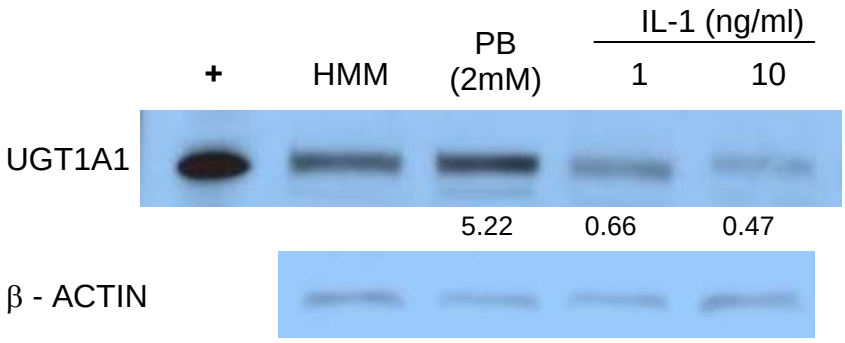
Hepatocytes in 3D culture system were treated with IL-1, IL-2, IL-6 and TNFα (0, 0.3, 1, 5 and 10 ng/ml) for 72 hours and the effect on UGT1A1 mRNA, protein expression and activity was determined.

Figure 39A shows that increasing concentrations of IL-1 significantly inhibited UGT1A1 mRNA expression. At the highest concentration of IL-1 (10 ng/ml), UGT1A1 mRNA expression was inhibited to 57.7 ± 3.4 % of the untreated control. Similarly, the UGT1A1 protein expression was reduced to 66 % and 47 % of the control value, at IL-1 concentrations 1ng/ml and 10 ng/ml, respectively (Figure 39B). This decrease in UGT1A1 mRNA and protein expression was reflected in a decreased formation rate of estradiol-3-glucuronide (E-3-G). Figure 39C shows that E-3-G formation rate was decreased to 41.2 ± 3.1 % of control value, at 10 ng/ml of IL-1.

A.



B.



C.

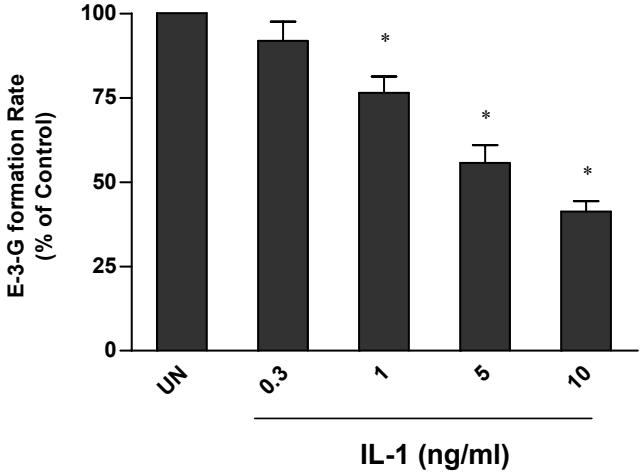


Figure 39. Effect of IL-1 on UGT1A1 mRNA expression, protein content and activity

Hepatocytes were exposed to IL-1 (0-10 ng/ml) for 72 hours and UGT1A1 A) mRNA expression, B) protein content and C) activity were determined. The figure shows the mean of values obtained from three livers, with the S.D. indicated by the vertical bars. All mRNA values are normalized to GAPDH while and protein values are normalized to β-actin expression. *, significantly different from control cells, $p \leq 0.05$.

IL-2 at concentrations 0.3 ng/ml and 1 ng/ml showed 1.27-fold and 1.23-fold increase in UGT1A1 mRNA expression, respectively (Figure 40A). IL-2 did not show any significant effect on UGT1A1 protein content (Figure 40B), while UGT1A1 activity was inhibited significantly only at 10ng/ml. The formation rate of E-3-G was 83.2 ± 5.2 % of control after treatment with 10 ng/ml IL-2 (Figure 40C).

Surprisingly, IL-6 at higher concentrations showed significant increase in UGT1A1 mRNA expression. This increase was 1.7 ± 0.2-fold and 1.6 ± 0.3-fold compared to control, at 5ng/ml and 10 ng/ml IL-6, respectively (Figure 41A). UGT1A1 protein content, on the other hand, was decreased to 88 % and 53 % of control after exposure to IL-6 at concentration 1ng/ml and 10 ng/ml (Figure 41B). Similarly, formation rate of E-3-G was decreased to 88.3± 5.3 %, 82.1± 4.8 % and 72.7± 4.2 % of control value, at 1 ng/ml, 5 ng/ml and 10 ng/ml of IL-6 (Figure 41C).

TNFα treatment did not significantly alter UGT1A1 mRNA expression, but decreased the UGT1A1 protein content to 77 % and 54 % of control at 1 ng/ml and 10 ng/ml, respectively (Figure 42A and Figure 42B, respectively). But, with increasing concentrations of TNFα, the formation rate of E-3-G was decreased up to 76.5 ± 3.6 % of control, at 10ng/ml (Figure 42C).

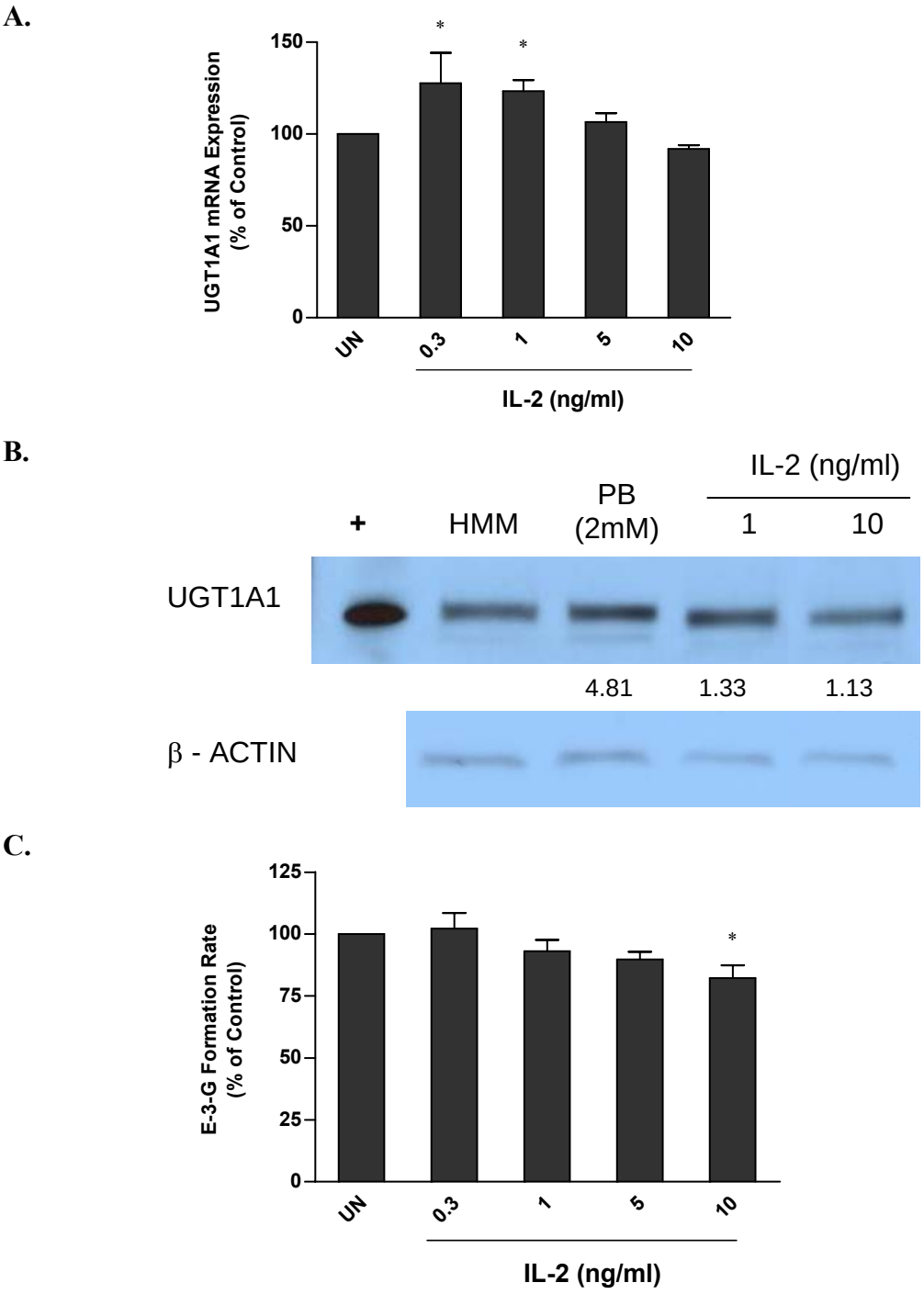
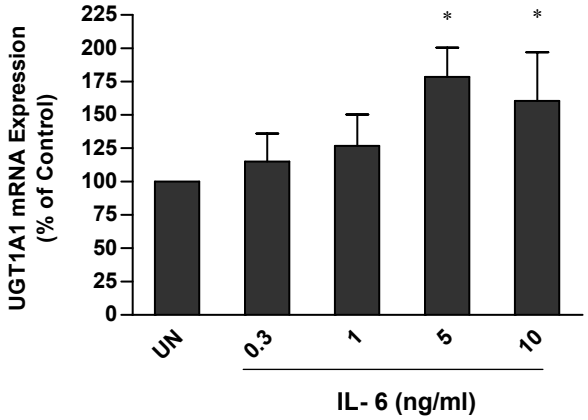


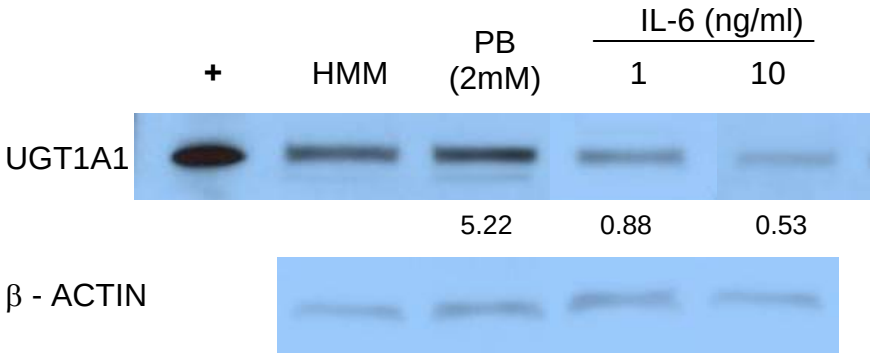
Figure 40. Effect of IL-2 on UGT1A1 mRNA expression, protein content and activity

Hepatocytes were exposed to IL-2 (0-10 ng/ml) for 72 hours and UGT1A1 A) mRNA expression, B) protein content and C) activity were determined. The figure shows the mean of values obtained from three livers, with the S.D. indicated by the vertical bars. All mRNA values are normalized to GAPDH while and protein values are normalized to β-actin expression. *, significantly different from control cells, $p \leq 0.05$.

A.



B.



C.

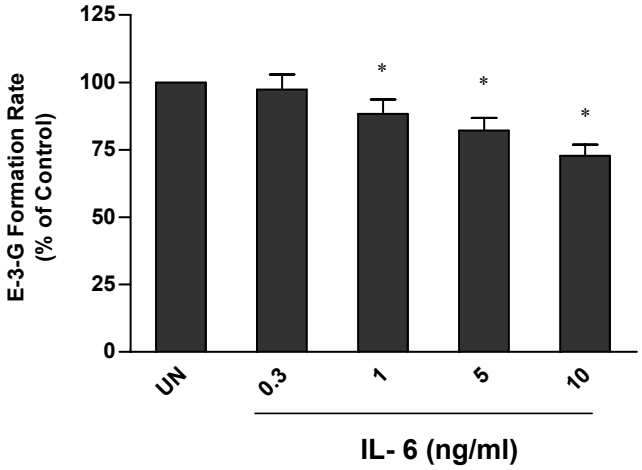


Figure 41. Effect of IL-6 on UGT1A1 mRNA expression, protein content and activity

Hepatocytes were exposed to IL-6 (0-10 ng/ml) for 72 hours and UGT1A1 A) mRNA expression, B) protein content and C) activity were determined. The figure shows the mean of values obtained from three livers, with the S.D. indicated by the vertical bars. All mRNA values are normalized to GAPDH while and protein values are normalized to β-actin expression. *, significantly different from control cells, $p \leq 0.05$.

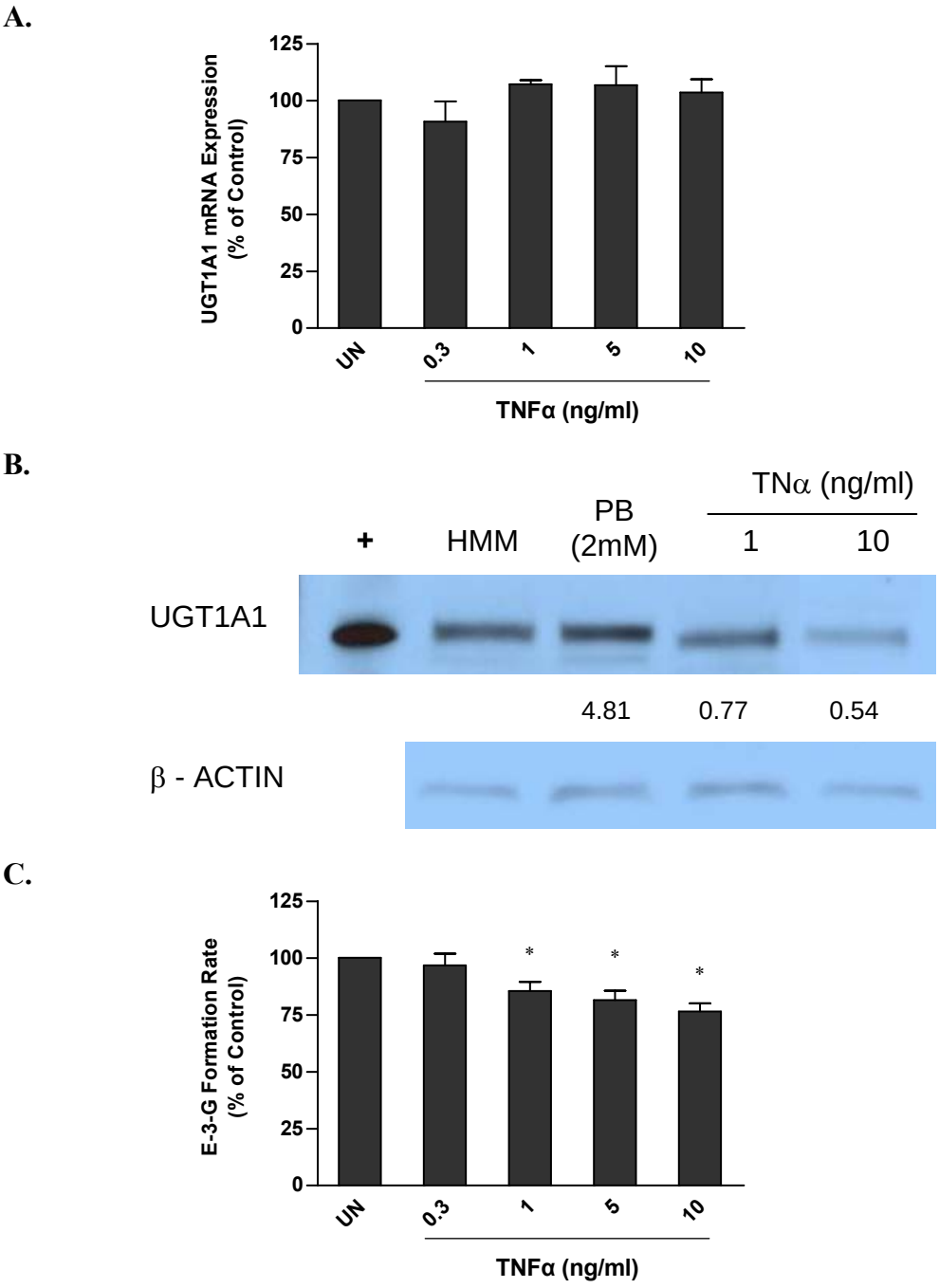


Figure 42. Effect of TNFα on UGT1A1 mRNA expression, protein content and activity

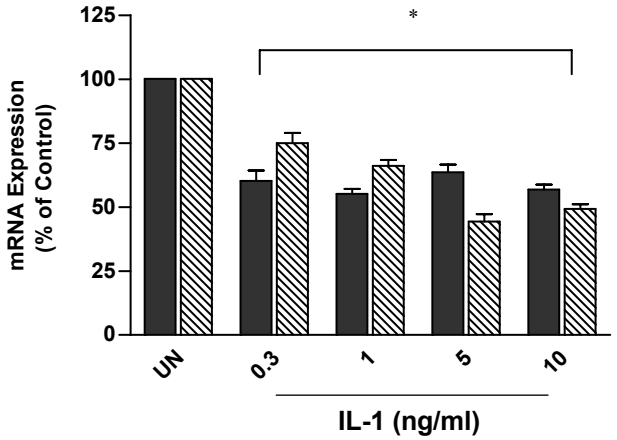
Hepatocytes were exposed to TNFα (0-10 ng/ml) for 72 hours and UGT1A1 A) mRNA expression, B) protein content and C) activity were determined. The figure shows the mean of values obtained from three livers, with the S.D. indicated by the vertical bars. All mRNA values are normalized to GAPDH while and protein values are normalized to β-actin expression. *, significantly different from control cells, $p \leq 0.05$.

7.5.2. Effect of cytokines on mRNA expression of PXR and CAR

The mRNA expression of PXR and CAR was determined using RT-PCR in the cells treated with IL-1, IL-2, IL-6 and TNFα (0, 0.3, 1, 5 and 10 ng/ml) for 72 hours.

Figure 43A shows that mRNA expression of both PXR and CAR was decreased to 56.8 ± 2 % and 49.3 ± 3 % of control, respectively, at 10 ng/ml IL-1 concentration. IL-2 did not show any changes in PXR and CAR at lower concentrations, but showed a significant decrease in PXR and CAR expression at higher concentrations (10 ng/ml). The PXR and CAR expression was decreased to 83 ± 2 % and 81 ± 3 %, respectively, at 10 ng/ml IL-2 (Figure 43B). Figure 44A shows that mRNA expression of both PXR and CAR was increased to 1.34 ± 0.07 % and 1.9 ± 0.03 % of control, respectively, at 10 ng/ml IL-6 concentration. TNFα did not show any changes in PXR and CAR concentrations ≤ 1ng.ml, while mRNA expression of PXR was increased 1.5-fold over control at TNFα (10 ng/ml) (Figure 44B).

A.



B.

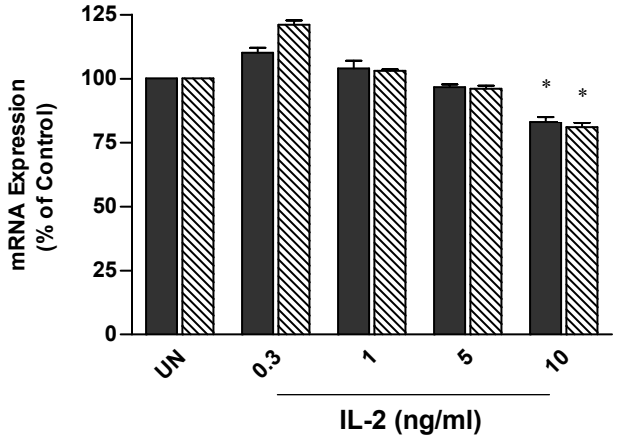
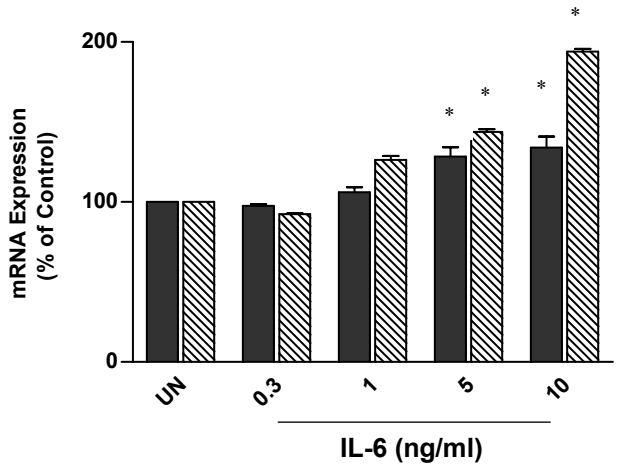


Figure 43. Effect of IL-1 and IL-2 on mRNA expression of PXR and CAR

Hepatocytes were exposed to A) IL-1 and B) IL-2 (0-10 ng/ml) for 72 hours and the mRNA expression of PXR (closed bars) and CAR (thatched bars) was determined. The figure shows the mean of values obtained from three livers, with the S.D. indicated by the vertical bars. All mRNA values are normalized to GAPDH expression. *, significantly different from control cells, $p \leq 0.05$.

A.



B.

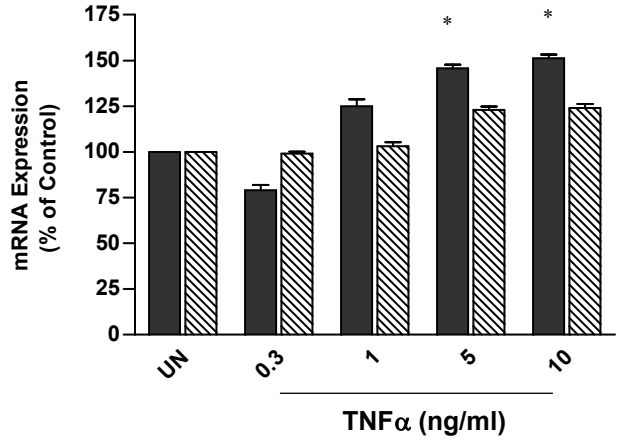


Figure 44. Effect of IL-6 and TNFα on mRNA expression of PXR and CAR

Hepatocytes were exposed to A) IL-6 and B) TNFα (0-10 ng/ml) for 72 hours and the mRNA expression of PXR (closed bars) and CAR (thatched bars) was determined. The figure shows the mean of values obtained from three livers, with the S.D. indicated by the vertical bars. All mRNA values are normalized to GAPDH expression. *, significantly different from control cells, $p \leq 0.05$.

7.6. Discussion

In humans, 16 different UGT isoforms have been classified into either 1A or 2B subfamilies (Tukey and Strassburg, 2000). Among the UGT family, UGT1A1 is involved in the glucuronidation of various endogenous substrates such as bilirubin and estradiol, and drugs like acetaminophen, thus making it the most important UGT isoform (Cheng et al., 1998; Court et al., 2001). Limited data have been published on the effects of pro-inflammatory cytokines on the expression and activity of UGT1A1. Small decreases in hepatic UGT activity were reported after administration of lipopolysaccharides (LPS) in rats. In isolated pig hepatocytes, IL-1α and TNFα caused an early inhibition of glucuronidation (Monshouwer et al., 1996). In a single study with human liver biopsies, decreased UGT1A4, UGT2B4 and UGT2B7 mRNA levels were observed with increased inflammation (Congiu et al., 2002). Most studies have used rat and pig livers and are limited due to analysis of UGT mRNA levels only or activity only.

In the present study we have used human hepatocyte cultures to study the effect and dose dependency of IL-1, IL-2, IL-6 and TNFα on UGT1A1 expression and activity. Hepatocytes in culture provide an intact system containing all the necessary cofactors for oxidative, reductive and conjugative metabolism as well as all the various regulatory elements required to maintain and modulate the expression of the enzymes.

UGT1A1 catalytic activity was determined by measuring the formation rate of estradiol-3-glucuronide. The conjugation of the phenolic 3-OH group of estradiol has been reported to be mediated mainly by UGT1A1 in humans (Senafi et al., 1994; Soars et al., 2004). Therefore, estradiol was chosen as a probe substrate to measure UGT1A1 activity.

In the present study we have shown that IL-1 (0-10 ng/ml) down regulates UGT1A1 expression and activity, in a concentration dependent manner. Our observations are consistent

with the report by Monshouwer et. al., (1996) where IL-1 (10-1000 U/ml) inhibited the UDPGT-dependent enzyme activities by more than 20- 60% when pig hepatocytes were exposed to IL-1 for 12 hours. Treatment of human prostate cells, LNCaP cells, with IL-1 has been reported to inhibit dihydrotestosterone (DHT) glucuronidation by 70% (Levesque et al., 1998). In contrast a study by Strasser et. al., (1998) has reported that exposure of rat hepatocytes to IL-1 in a concentration range of 5-50 U/ml did not result in any significant alterations in UGT1*1 or UGT2B3 mRNA levels.

Downregulation of UGT1A1 mRNA expression along with decreased protein content and activity after IL-1 treatment suggests that IL-1 possibly acts by altering the transcriptional process. PXR and CAR are the transcriptional factors involved in the regulation of UGT1A1 (Mackenzie et al., 2003). In the present study, IL-1 decreased the mRNA expression of both PXR and CAR to 50% of control value. This supports our hypothesis that IL-1 downregulates UGT1A1 by altering the mRNA expression of nuclear receptors, PXR and CAR that control expression of UGT1A1.

IL-2 showed a 1.2–fold increase in UGT1A1 mRNA expression at lower concentration, while there was no effect at higher concentration. Also, IL-2 did not alter the UGT1A1 protein content at the concentrations used in the study. UGT1A1 activity remained unchanged when hepatocytes were exposed to IL-2 concentrations ≤ 5 ng/ml and was inhibited to 83.2 % of control at 10 ng/ml IL-2. There are no published studies documenting the effect of IL-2 on UGT1A1 expression and activity. Interleukin-2 (IL-2) in rat primary hepatocytes downregulates CYP3A activity within 24 hours, with only transient suppression of CYP3A4 activity in human hepatocytes (Tinel et al., 1999). Sunman et. al. (2004) have shown no sustained suppression of CYP3A activity with IL-2 in human hepatocyte cultures. However, in hepatocyte/Kupffer cell

cocultures, a concentration dependent 50-70% decrease in CYP3A activity was observed with IL-2 at 72 hours. This suggests that IL-2 mediates its inhibitory effect 'indirectly' through Kupffer cells by mediating release of IL-1, IL-6 or TNFα which in turn downregulate CYP3A4 activity. Failure of IL-2 to show any significant suppression of UGT1A1 in our experiment may be attributed to the absence of Kupffer cells in the model system used.

In our experiments, IL-6 decreased UGT1A1 protein content to 53% - 88% and activity to 72.2 % - 88.3% of control at concentrations greater than 1 ng/ml. Surprisingly, at higher concentrations IL-6, UGT1A1 mRNA expression was increased up to 1.7-fold of control. Monshouwer et. al., (1996) have reported only 20% inhibition of UDPGT-dependent enzyme activities after IL-6 treatment (1000 U/ml) in pig hepatocytes. In rat hepatocytes, IL-6 has been shown to suppress the mRNA expression of UGT1A1 to 77% of control and UGT2B3 to 60% of control (Strasser et al., 1998). In the present study, the mRNA expression of transcriptional factors PXR and CAR were also measured in IL-6 treated cells. PXR mRNA was increased 1.3-fold, while CAR mRNA expression was increased 1.9-fold over control value, at 10 ng/ml IL-6. Increased mRNA expression of UGT1A1 along with increased PXR and CAR expression with decreased protein content and UGT1A1 activity suggests that IL-6 mediated suppression of UGT1A1 may be associated with post transcriptional changes. The exact mechanism by which IL-6 decreases UGT1A1 activity cannot be explained by the present data.

TNFα did not significantly alter UGT1A1 mRNA expression, but with increasing concentrations of TNFα, the UGT1A1 protein content and formation rate of E-3-G was decreased to 54 % and 75 % of control, respectively. The mRNA expression of PXR and CAR remained unchanged after TNFα treatment but increased at highest concentration used. This suggests that possibly TNFα mediated its effect on UGT1A1 enzyme by affecting the

translational processes such as affecting the stability of protein. There is only one study in pig hepatocytes, where TNFα (10 –1000 U/ml) has been shown to mediate a 20- 60 % reduction in UDPGT-dependent enzyme activities at a 12-hour treatment period (Monshouwer et al., 1996).

In summary, this is the first study to the best of our knowledge documenting the effect of IL-1, IL-2, IL-6 and TNFα on human UGT1A1 expression and activity in human hepatocytes. Given that previous studies were carried out in rat or pig hepatocytes or in artificial cell culture systems, our observations provide important insight into the differential effect of cytokines on UGT1A1 expression and activity. All four cytokines inhibited the formation rate of estradiol-3-glucuronide thus inhibiting UGT1A1 activity to different extent. The rank order of inhibition potency was IL-1 >IL-6 ≅ TNFα > IL-2, with maximum inhibition seen after IL-1 treatment. The lowered UGT1A1 activity during the inflammation process can decrease the conjugation of bilirubin and increase the concentration of bilirubin in bile and serum. The metabolism of drugs that are conjugated by UGT1A1 will be decreased during inflammation and dosing adjustments of such drugs are necessary in patients. Our observations indicate that cytokines contribute at most a 2-fold change in activity of UGT1A1. Other factors may be of more importance in the observed variation in glucuronide conjugation in liver transplant patients. The overall effect of cytokines on UGT1A1 in general appears to be modest downregulation of this enzyme.

8. Discussion and summary

8.1. Discussion and Summary

The objective of the work carried out in this dissertation was to evaluate the effect of endogenous mediators such as cytokines and exogenous chemicals such as HIV protease inhibitors on hepatic drug metabolizing enzymes and transporters. In this research work, we systematically studied the effect of clinically used HIV-protease inhibitors on phase I and phase II metabolizing enzymes as well as on drug transporters. Extending earlier findings in our laboratory, we also investigated the effect of cytokines on phase II enzymes and drug transporters. Our findings have been used to postulate and predict potential mechanisms responsible for modulation of drug metabolizing enzymes and transporters. Several key findings were generated in this work, which are summarized in the following section.

We have used primary cultures of human hepatocytes (PCHH), which provide several advantages over other systems used to study drug metabolism and drug transporters. The PCHH contain all the necessary cofactors for oxidative, reductive and conjugative metabolism as well as various regulatory elements required to maintain and induce/inhibit the expression of enzymes. The PCHH system is also well suited to study the role of nuclear receptors such as pregnane X receptor (PXR) and constitutive androgen receptor (CAR), which are involved in the regulation of metabolizing enzymes and hepatic transporters.

In the first part of this study, we found a concentration dependent effect of individual protease inhibitors on CYP3A4 expression and activity in a human hepatocyte system. mRNA expression, protein content and activity were used as key metrics to determine the interaction potential of the protease inhibitors with different CYP3A4 substrates. The interaction potential of different protease inhibitors was determined to be widely different and a relative ranking of this interaction potential has been generated. We have also reported that the CYP3A4 inhibition or

induction by protease inhibitors after treatment and removal is a slowly reversible over time. The kinetic parameters such as $t_{1/2}$ and recovery time for CYP3A4 expression and activity, after drug discontinuation, have been calculated. These parameters can quantify the effect of protease inhibitors on CYP3A4 and may prove useful in further studies. The findings from this study thus can provide insight into the clinically observed complex drug-drug interactions with other CYP3A4 substrate drugs.

In the second part of this work, we have studied the effect of ritonavir, indinavir and amprenavir on the expression and activity of the UGT1A1 enzyme. This is the first study to the best of our knowledge, to report the effect of increasing concentrations of protease inhibitors on UGT1A1 mediated metabolism in human hepatocytes and to document EC_{50} values for UGT1A1 induction after RTV treatment. We also demonstrated that the effect of ritonavir and amprenavir on UGT1A1 is 'indirect' and is possibly mediated through the nuclear receptors, while the inhibitory effect of indinavir on UGT1A1 activity is direct. This study shows that protease inhibitors can significantly modulate the expression and activity of UGT1A1 thereby affecting the clearance of drugs and endogenous compounds metabolized by glucuronidation. In contrast to the effect of inhibition of CYP3A, HIV-protease inhibitors generally appear to moderately induce UGT1A1.

The third part of this work aimed to study the effect of HIV-protease inhibitors on drug efflux transporters. This is among the first few studies in the human hepatocyte system to report that increasing concentration of HIV-protease inhibitors have differential effects on expression of drug efflux transporters such as P-glycoprotein, MRP2, MRP6 and BSEP. By monitoring and measuring the concomitant regulation of PXR and CAR expression during HIV-protease inhibitor treatment, we have postulated that transporter expression is mediated via mechanisms

involving nuclear receptors such as PXR and/or CAR for MDR1, MRP2 and BSEP and other nuclear receptors as in the case of MRP6. Knowing the effects of HIV-protease inhibitors on the expression and activity of hepatic transporters, such as those studied in this work may hence prove highly beneficial in understanding the pharmacokinetic behavior of drugs that are transported via P-glycoprotein, MRP2, MRP6 and BSEP.

Another objective of this research work was to determine the effect of individual cytokine exposure on hepatic drug transporter expression and activity. We studied the concentration and time dependence of cytokine exposure on BSEP expression and activity. It was shown that maximum suppression in mRNA expression and BSEP activity occurs after 72 hours of cytokine treatment. Different cytokines exhibit different inhibition potential which can be ranked as IL-1 ≅ TNFα > IL-6 > IL-2 in decreasing order of inhibition. These findings indicate that any alteration of above-cited cytokines in deceased states such as infection, cancer or rejection after transplantation will modulate the expression and activity of hepatic BSEP and thus impact the pharmacokinetics of substrate drugs and/or bile secretion leading to cholestasis.

The last part of this dissertation aimed to study the effect of IL-1, IL-2, IL-6 and TNFα on UGT1A1 expression and activity in human hepatocytes. Most of the studies reported in literature have been carried out in rat or pig hepatocytes or in artificial cell culture systems. Our study is among the first studies conducted on human hepatocytes and thus provides important insight into the differential effect of cytokines on UGT1A1 expression and activity. All four cytokines inhibited formation rate of estradiol-3-glucuronide thus inhibiting UGT1A1 activity to a different extent. The rank order of inhibition potency was IL-1 >IL-6 > TNFα > IL-2, with maximum inhibition seen after IL-1 treatment. The lowered UGT1A1 activity during inflammation process can decrease the conjugation of bilirubin and increase the concentration of

bilirubin in bile and serum. The metabolism of drugs that are conjugated by UGT1A1 will be decreased during inflammation thereby necessitating the dosing adjustments of such drugs in patients.

8.2. Clinical Implications

1. HIV-protease inhibitors will contribute to the observed variability in the pharmacokinetics of immunosuppressive drugs to the maximum extent of all the variables tested in our laboratory. This effect is primary mediated through inactivation of CYP3A enzymes. Significant reduction in the doses of all substrates of CYP3A4 is necessary in transplant patients receiving concomitant HIV-protease inhibitors.
2. The inhibitory effect of HIV-protease inhibitors is immediate (based on previous studies in our laboratory). Significant decrease in doses of CYP3A4 substrate drugs is necessary immediately after the addition of HIV-protease inhibitors to the therapeutic regimen in transplant patients. However, the recovery of inhibition of CYP3A is time dependent. It will take approximately 8-10 days for complete recovery of inhibition of CYP3A. Specific attention must be paid to the dosing regimen of CYP3A substrates during this time period.
3. The contribution of cytokines to the observed variability in CYP3A mediated metabolism has been previously reported in our laboratory to be modest at best. Levels of CYP3A enzymes will be modestly decreased in the presence of elevated cytokines.
4. With regard to the variability in activity of UGT1A1, HIV-protease inhibitors will modestly contribute to the observed variabilility in the pharmacokinetics of the drugs that are conjugated by this enzyme. In contrast to the effect of inhibition of CYP3A, HIV-protease inhibitors will increase expression and activity of possibly UGT1A1 through the

induction of PXR and CAR. Dosing regimen of drugs metabolized by UGT1A1 must be increased in the presence of HIV-protease inhibitors.

5. The inhibitory effect of cytokines on UGT1A1 appears to be modest. Similar to their effect on CYP3A, cytokines decreased the expression and activity of UGT1A1. Decreased dosing of UGT1A1 substrates is necessary in patients with elevated inflammatory cytokine levels.
6. The effect of HIV-protease inhibitors on transporters is modest as compared to their effects on CYP3A. Due to their effect on PXR and CAR, HIV-protease inhibitors tend to increase expression of certain transporters. The uptake and efflux of the substrates of the transporters will be higher in patients taking HIV-protease inhibitors. The effect of HIV-protease inhibitors on CYP3A is likely to override the effects of HIV-protease inhibitors on transporters like P-glycoprotein.
7. Consistent with the effect on CYP3A and UGT1A1, cytokines decrease expression and activity of several transporters. The effect of cytokines appears to be greater on transporters than on CYPs and UGTs. This requires decreased dosing of drugs during elevated cytokine levels.

8.3. Limitations and Future directions

1. While this is the first report of a systematic evaluation of HIV-protease inhibitors and cytokines on phase I enzymes, phase II enzymes and transporters, all the studies were carried out in primary cultures of human hepatocytes. Coculture using Kupffer cells will provide additional information that may be more relevant to *in vivo* situation, especially with regards to the effect of cytokines, as Kupffer cells play an important role *in vivo* during inflammatory process.

2. The observations of this study are limited to CYP3A and UGT1A1. Additional studies need to document the effect of HIV-protease inhibitors and cytokines on other drug metabolizing enzymes.
3. While we evaluated the activity of BSEP only, activity of other hepatic transporters need to be evaluated in future studies using specific substrates for transporters such as bromosulphthalein for MRP2 and rhodamine123 for P-glycoprotein.
4. While effects of individual cytokines were studied, future studies should include combination of cytokines to determine potential additive or synergistic effects.

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